

The Mechanism of Cell Cycle Arrest in the ERCC1-Deficient Mouse Model

Fátima Núñez Mangado



Ph.D.
University of Edinburgh
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A mis padres

Abstract

ERCC1 is a gene essential for the repair process of UV-induced DNA damage known as Nucleotide Excision Repair (NER). Deficiencies in this DNA repair mechanism have been associated with the skin cancer prone human syndrome Xeroderma Pigmentosum (XP) and also with Cockayne's Syndrome (CS) and Trichothiodystrophy (TTD). The main role of RERCC1 is to act as a NER endonuclease but it has also been proposed that it may have a role in recombination.

The ERCC1 knock out (k/o) mouse model was first described by McWhir *et al.* in 1993¹. McWhir and co-workers described ERCC1-deficient mice as being severely runted, dying before weaning at approximately three weeks of age from hepatic failure, having severe aneuploidy and polyploidy in their liver and presenting elevated levels of p53 in this tissue. This k/o model shared no similarities with any of the already known NER deficiency-associated syndromes which led to the theory that a deficiency in the other non-NER ERCC1 function(s) may be responsible for the observed differences. The aim of this project was to gain a better understanding of the precise mechanisms behind this atypical phenotype. Indeed, studies carried out on both liver tissue and primary fibroblast material have resulted in the development of a theory for the ERCC1 k/o phenotype.

ERCC1-deficient and wild type primary fibroblasts showed the same response to UV damage in terms of DNA replication arrest after UV. This behaviour, however, differed significantly from that observed in human cells. ERCC1-deficient cells were shown both to induce p53 with lower UV doses than their wild type counterparts and to induce the cyclin-dependent kinase inhibitor, p21, in response to UV in a p53-independent manner. From the liver tissue studies a number of conclusions can be derived which are outlined below.

Levels of p21 transcript were also shown to be elevated in ERCC1 deficient livers and this increase of p21 was p53-independent. In order to get a more accurate picture of the cell cycle status in these hepatocytes the DNA synthesis levels as measured by the BrdU incorporation rates and cycling status were studied. Hepatocytes of ERCC1-deficient animals were shown to have reduced levels of DNA synthesis when compared to their wild type counterparts and, through the combination of BrdU, Ki67 and centromeric staining, it was also concluded that the most likely explanation for the aneuploidy and polyploidy we see in these livers is that these cells are arrested in G2. Another point that emerged when morphometric studies were carried out in wild type, ERCC1-/-, p53-/- and p53/ERCC1 double null livers, was that binucleation is repressed in the ERCC1 k/o hepatocytes. Although the absence of p53 does not rescue the liver phenotype that is seen in the ERCC1 k/o mice, it results in the bypass of the repression of binucleation, as shown by wild type levels of binucleate hepatocytes in ERCC1/p53 double null livers.

When all this data is taken together it is possible to think of a likely explanation for the observed ERCC1 k/o phenotype. Briefly, ERCC1-deficient livers are subject to

¹ McWhir, J. and Selfridge, J. and Harrison, D.J. and Squires, S. and Melton, D.W.(1993) Mice with DNA-repair gene (ERCC1) deficiency have elevated levels of p53, liver nuclear abnormalities and die before weaning. *Nature Genetics* 5:217-224

endogenous damage that cannot be repaired, this damage results in the activation of two separate pathways, one mediated by p53 and one mediated by p21. Severe, accelerated hepatocyte polyploidisation as a response to the existing endogenous damage results in the observed prematurely aged appearance of the liver. This together with an impairment of liver growth, probably mediated by p21 (as shown by elevated levels of hepatocytes arrested in G2), results in early hepatic failure and the observed premature death.

No human syndrome has yet been identified as being caused by a defect in ERCC1, so a subsidiary aim of this project was to see if any human condition could be attributed to an ERCC1 deficiency. Taking as a starting point the prematurely aged appearance of the ERCC1 k/o mice, a rare human premature ageing syndrome called Hutchinson-Gilford progeria (HGP) was selected as a possible candidate for an ERCC1-related human condition. Primary fibroblasts derived from a HGP patient showed decreased levels of ERCC1 protein, but no evidence for mutations in the ERCC1 gene was found. However, an impaired p53 response to UV-irradiation was observed. Overall, this work has opened the doors for the study of premature ageing in humans in relationship to NER and p53.

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Declaration

I hereby declare that I composed this thesis entirely myself and that it describes my own research, unless otherwise stated. The experiments were designed in collaboration with my supervisor Prof. David W. Melton.

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Chapter 1

Introduction

1.1 DNA Repair

1.1.1 Damaging agents

The DNA from living organisms is constantly exposed to various agents that affect its integrity by chemically or physically modifying its structure. Damage to DNA can be caused in a number of distinct ways that fall under one of the following two general categories: spontaneous or environmental in origin.

Amongst the first category are base mismatches, and abnormal base pairing occurring from deamination of bases (cytosine to uracil, adenine to hypoxanthine and guanine to xanthine). Such oxidative deamination can also result from the action of the mutagen nitrous oxide. Apurinic and apyrimidinic sites can occur spontaneously or through the action of DNA glycosylases on altered bases. Among the environmental factors are the ionizing radiation which can activate clastogenic factors that mediate the formation of breaks in either or both DNA strands, alkylating agents which are chemical agents that damage DNA by introducing bulky adducts and chemicals such as mitomycin C as well as UV irradiation which can damage DNA by introducing inter or intrastrand crosslinks that ultimately can impair subsequent transcription and translation processes (Friedberg et al., 1995).

Various single enzyme and complex multi-step DNA repair systems are operational in the cell to ensure proper functioning and faithful transmission of genetic information. Examples of the latter class of repair pathways are nucleotide excision repair (NER),

base excision repair, post-replication repair and recombination repair. These systems have in general a broad lesion specificity. For example, NER recognises and eliminates a wide spectrum of structurally unrelated lesions such as UV-induced photoproducts (cyclobutane pyrimidine dimers -CPDs- and (6-4) pyrimidine pyrimidones -(6-4)PDs-), (bulky) chemical adducts and certain types of crosslinks, whereas recombination repair is required for the removal of X-ray -induced double-strand breaks and interstrand crosslinks (Bootsma and Hoeijmakers, 1994).

1.1.2 NER

Ultraviolet light and ionizing radiations are often used as prototype DNA-damaging agents because of their ease of delivery to cells and their relative specificity for damaging nucleic acids versus other cellular components. These two types of genotoxic agents differ in the spectra of DNA damage they produce, and consequently the repair pathways that deal with them are also different. In mammalian cells the photoproducts (mainly cyclobutane pyrimidine dimers) caused by UV are repaired exclusively by the Nucleotide Excision Repair pathway (NER). NER is the most extensively studied repair system¹ and it processes damage by locating the lesions, excising an oligomer carrying the damaged nucleotides and synthesising a repair patch using the opposite strand as a template (Sancar and Tang, 1993; Hanawalt, 1995; Wood, 1997b).

The biological consequences of the UV lesions depend on the DNA sequence context, the relative efficiency of repair, and the particular end point (e.g., cell survival, mutation induction, or inhibition of DNA replication -see Replication section 1.2- or transcription). In addition, the same NER system that protects skin cells by removing UV photoproducts also acts on a number of bulky chemical adducts and may protect internal tissues against cancer (Thompson, 1998). Some of the interstrand crosslinks produced by agents such as mitomycin C, cisplatin and melphalan are probably repaired by a recombinational mechanism that requires certain components of the NER pathway and other, unidentified gene products (Thompson, 1998). Although NER can act as a 'backup' system for other repair pathways such as base excision repair, there is no backup system for NER. In addition, bulky lesions that remain unrepaired by the

¹ For a review on the subject see Sancar, 1993; Hanawalt, 1995; Wood, 1997b

time DNA is replicated are potentially mutagenic and have to be dealt with through other damage-tolerance mechanisms known as post-replication repair (see Replication section 1.2).

NER in *E. coli*

The NER mechanism is well understood in *Escherichia coli* in which six proteins UvrA, UvrB, UvrC, UvrD, polymerase I (Pol I) and ligase (Lig) are necessary and sufficient to carry out this process in vitro (Sancar and Tang, 1993). UvrA and UvrB form the UvrA2B complex which has helicase activity enabling it to bind to the DNA and move along it searching for abnormalities. On recognition of damaged DNA the Uvr A subunit dissociates, leaving UvrB bound at the site of damage. In this position, Uvr B recruits a Uvr C subunit, forming an incision complex. The single strand incisions occur seven nucleotides upstream of the lesion and three or four nucleotides downstream. Another protein, Uvr D acts along with Pol I to release a damage-bearing 12-13mer oligonucleotide and the bound Uvr complex. Finally, the resultant gap is filled in by Pol I and then ligated.

NER in eukaryotes

Less is known however, about the biochemistry of the NER process in eukaryotic cells. In the yeast *Saccharomyces cerevisiae* mutant analysis and gene cloning have demonstrated the existence of more than 10 genetic loci implicated in excision repair (Friedberg et al., 1995). These genes are defined by the RAD3 epistasis group.

Habraken and co-workers (Habraken et al., 1993) suggested that following the recognition of DNA lesions by RAD14, helicase activity of RAD3 and RAD25 enables a RAD1/RAD10 complex to access the site of damage, nicking the double stranded DNA. Subsequent helicase action produces a single strand which can then be incised by RAD2. This results in the removal of a lesion within an oligonucleotide.

The majority of the RAD genes that are required for the damage-specific incision of DNA during nucleotide excision repair are conserved in, and may be functionally related to NER proteins in other eukaryotes, including humans. Evidence in support

of this idea comes from studies with the yeast RAD10 and the human ERCC1 genes. Expression of Rad 10 protein in Chinese hamster mutant cells defective in ERCC1 results in partial complementation of both UV sensitivity and defective nucleotide excision repair. Similarly, the human XPD cDNA (see below) can rescue the lethality of a Rad 3 disruption mutation in yeast cells.

1.1.3 NER in mammalian cells: NER syndromes

The isolation of genes involved in nucleotide excision repair in bacteria and in yeast by phenotypic complementation of DNA damage-sensitive repair-defective mutants, lead to similar studies with mammalian cells. Two mammalian cell systems have been extensively studied in this regard. One consists of a series of rodent cell lines selected in the laboratory for defective nucleotide excision repair, following the mutagenesis of established cell lines (see below). The second consists of cell lines derived from three repair-defective human hereditary diseases² (Bootsma and Hoeijmakers, 1994), xeroderma pigmentosum (XP), Cockayne's syndrome (CS) and a photosensitive form of trichothiodystrophy (TTD).

xeroderma pigmentosum

XP, indicating dry or parchment skin with pigmentation abnormalities, is a prototype of a multigenic human disorder in which mutations in several genes cause similar phenotypes (Boulikas, 1996). There are seven complementation groups of naturally occurring XP cell lines XP-A to XP-G. Up to now, these groups can be explained on the basis of single-locus mutations rather than a corecessive inheritance model (Thompson, 1998). The severity of the repair deficiency varies amongst these groups, being least severe in groups D and E.

Clinical features

XP is an autosomal recessive disease which occurs at a frequency of 10^{-6} in the United States. XP patients have reduced ability to repair the damage induced by UV

² The data presented in this section regarding NER syndromes has been taken from Thompson, 1998. Refer to this review for additional references. Additional comprehensive reviews on the subject can be found in Bootsma, 1994

light (Cleaver, 1968), which results in skin cancers on sun-exposed body sites at very early ages (before the age of 20, with a median age of onset of 8 years). A variety of complications are associated with the sun-exposed areas of the skin (see Table 1.1 and Fig. 1.1). The eyes and the tip of the tongue are often affected (Kraemer et al., 1987; Kraemer et al., 1994). The onset of both early dermatological symptoms of XP and tumour growth is correlated with the extent of DNA repair deficiency as measured by cell survival curves and incision activity (Thompson, 1998).

Neurological dysfunction is present in 20% of XP patients (XP-A > XP-D = XP-G) and is progressive. It includes hyporeflexia, sensorineural deafness, mental retardation, and microcephaly, which are associated with neuronal degeneration from loss of neurons, specially in the cerebellum and cerebral cortex (Thompson, 1998). These abnormalities are sometimes associated with slow growth and delayed secondary sexual characteristics. The neurological dysfunction also seems to be correlated to the degree of repair deficiency and is thought to arise from unrepaired oxidative damage in the brain.

Cellular features

Cells derived from XP patients are hypersensitive to killing by UV radiation or chemicals that produce bulky adducts. Hypersensitivity to UV varies widely both within and amongst complementation groups and also, the XP cells are more prone to UV-induced neoplastic transformation than normal cells.

A DNA repair deficiency in XP cells was first demonstrated by Cleaver in 1968 using repair synthesis and autoradiography (Cleaver, 1968). The phenotypic heterogeneity (different levels of repair synthesis) seen amongst the XP fibroblasts prompted genetic complementation analysis. Seven NER complementation groups were found to exist, which implied that many gene products were required to carry out the repair process that is deficient in XP cells. What is more, the residual repair in XP-C cells is clustered in a small fraction of the genome, now known to contain active genes (Thompson, 1998). Also, some XP-G cells show sensitivity to ionizing radiation, which appears to derive from a defect in repairing oxidative damage (Thompson, 1998).

XP patients show a high incidence of mutations, particularly in the skin tumours,

<i>Characteristics</i>	<i>XP</i>	<i>TTD</i>	<i>CS</i>
Clinical features			
Cutaneous photosensitivity	++ ^a	- or +	+
Pigmentation disturbances	+ ^a	-	-
Ocular abnormalities (sunlight)	++	-	-
Oral abnormalities	+	-	-
Actinic keratoses	+	-	-
Basal and squamous cell carcinomas, melanomas	++	-	-
Neurological abnormalities ^b	- or +	+	++
Slow rate of growth	- or +	+	++
Impaired sexual development	- or +	+	+
Sensorineural deafness	- or +	+	++
Mental retardation	- or +	+	++
Ichthyosis (fish-like scaly skin)	-	++	-
Brittle hair	-	++	-
Peculiar facies	-	+ ^c	+ ^c
Cataracts/pigmentary retinopathy	-	+	++
Dental caries	-	+	++
Cellular features			
Intrinsic chromosomal instability	-	-	-
Hypermutability	+	- or +	+
Excision repair in bulk DNA ^d	+	- or +	-
Excision repair in transcription-dependent repair	+	- or +	+

Table 1.1: Comparison of clinical and cellular features of XP, TTD and CS.

^aTraits that are hallmarks of the disease are indicated by (++), and traits that are sometimes associated with the disorder are indicated by (+); unassociated traits are shown by (-).

^bThese features are present in about 20% of XP patients. The molecular basis of the neurological dysfunction in XP differs from that of CS and TTD (see main text).

^cThe facial abnormalities of TTD and CS overlap in term of protruding ears. TTD often has a receding chin and a small thin or beaked nose, whereas CS tends to have large nose and projecting jaw.

^dXP-C is exceptional in being deficient in repairing bulk DNA, but proficient in transcription coupled repair.

This Table has been adapted from Thompson, 1998.

affecting mainly the p53 tumour suppressor gene. This is a direct effect of the mutagenic consequences of failing to repair DNA damage (Thompson, 1998). The variant form of XP (XP-V), although clinically indistinguishable from other XP groups, is normal for excision repair and forms a single complementation group. Apparently, XP-V has a reduced ability to elongate nascent DNA strands when replicating past

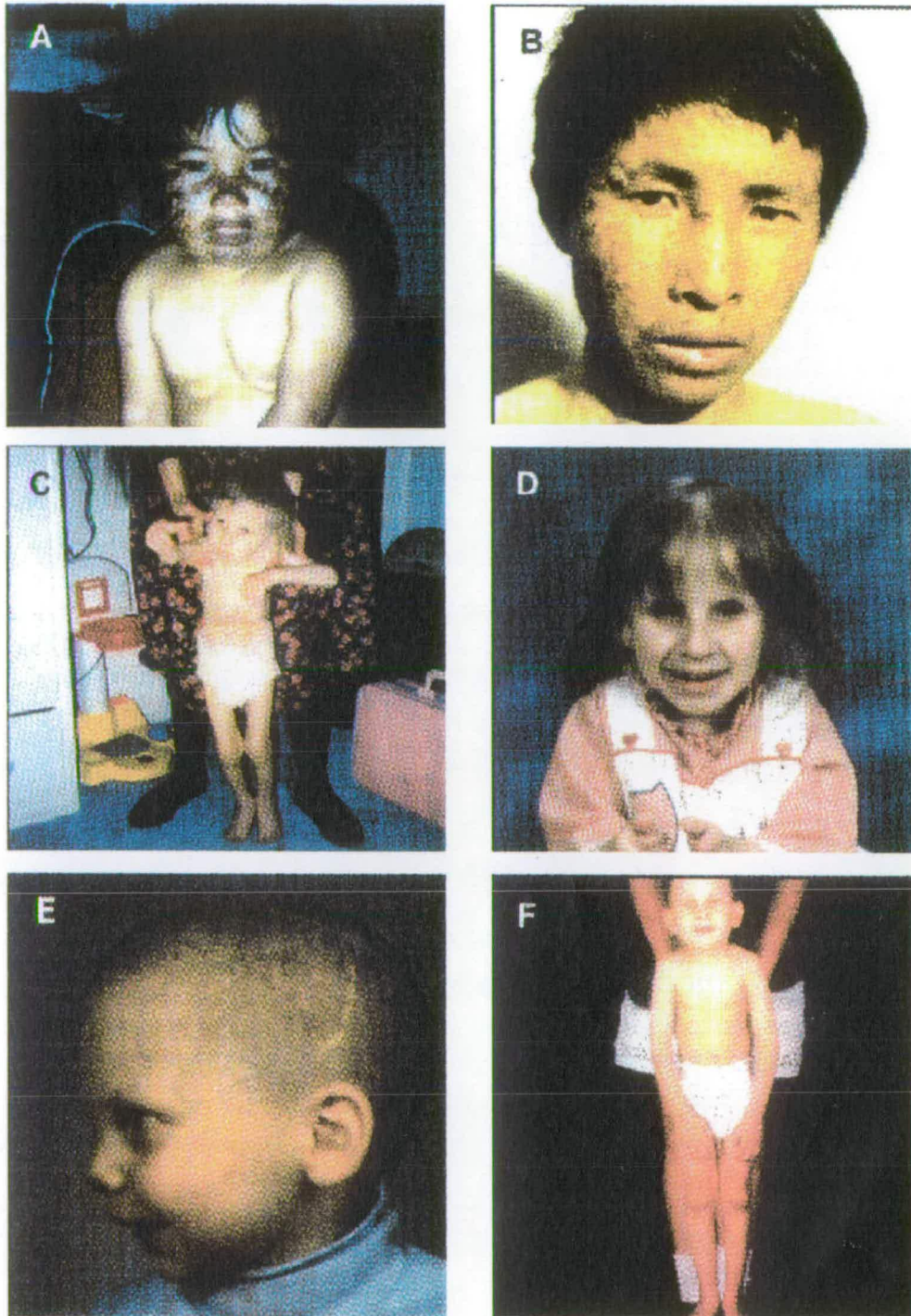


Figure 1.1: (A,B) Two XP patients (undefined complementation group); the patient in panel A subsequently showed decline of the central nervous system. (C,D) A CS patient at ages 4 and almost 7 years, respectively. (E,F) Two TTD patients. Taken from Thompson, 1998.

UV damage. XP-V cells are only slightly sensitive to killing by UV radiation but they are several fold sensitive to mutation induction and transformation by UV. At the molecular level, XP-V cells show error prone DNA replication in the presence of DNA damage (Thompson, 1998).

trichothiodystrophy

Clinical features

TTD is an autosomal-recessive disorder characterised by sulfur-deficient brittle hair, scaly skin, and physical and mental retardation. Patients may also have an unusual facial appearance, and altered body proportions. TTD comprises a variety of rare sulfur-deficient brittle hair disorders. Within them, the photosensitive form of TTD has been named PIBIDS (Photosensitivity, Ichthyosis, Brittle hair, Impaired intelligence, Decreased fertility, and Short stature). Approximately 50% of TTD patients show severe photosensitivity linked to deficient excision repair like that seen in XP cell lines, particularly XP-D. However, TTD patients do not show the same sort of skin abnormalities associated with XP (see Table 1.1 and Fig. 1.1), and there is no predisposition to skin cancer associated with TTD. Another difference between TTD and XP patients is the origin of the neurological abnormalities, which in TTD is predominantly due to demyelination. Prenatal diagnosis for TTD is available based on defective DNA repair in families known to carry mutant alleles (Thompson, 1998).

Repair deficiency

Complementation analysis of TTD cell lines found that most UV-sensitive TTD lines fall within the XP-D complementation group. Indeed, the cloned XPD gene was found to be able to correct the repair deficiency in the TTD cells. This could only be explained when the XPD gene was found to play a role in both NER and transcription.

TTD cell lines are characterised by a broad genetic heterogeneity (Thompson, 1998) and recently two more complementation groups of TTD have been identified. One of them corresponds to the XP-B group and the other has been shown to be defective in the TTD-A protein which is part of the basal transcription factor TFIID (Thompson, 1998).

Cockayne's syndrome*Clinical features*

CS patients suffer from postnatal growth failure and progressive neurological dysfunction, with frequent involvement of cutaneous photosensitivity, deafness, cataracts, pigmentary retinopathy and dental caries. In addition a dwarfed appearance, disproportionately long arms and sunken eyes. This disease has the appearance of premature ageing with a median life span of 12 years. CS patients do not show predisposition to skin cancer or the skin abnormalities associated with XP, despite showing sun sensitivity. As with TTD, CS patients' neurological abnormalities are due to demyelination in nerve tissue (see Table 1.1 and Fig. 1.1). In a few rare individuals, features of both CS and XP are found (XP-CS). The association of CS has been reported for XP-B, -D and -G groups.

Repair deficiency

CS cells are hypersensitive to killing by UV radiation and bulky adducts mutagens, although their sensitivity is approximately four- to eight-fold, which is less than the most sensitive XP cell lines. CS have also been recently reported to show moderate sensitivity to ionizing radiation. Although repair synthesis and dimer removal are normal in the bulk DNA of CS cells after UV radiation, the recovery of DNA synthesis seen in normal cells fails to occur in CS cells. CS cells also fail to recover RNA synthesis, which pointed toward a defect in these cells in the repair of DNA damage in transcribing regions of DNA. Cell fusion experiments with more than 30 CS cell lines have identified two complementation groups that are distinct from XP (CS-A and CS-B). Most patients belong to the CS-B group.

CS-A and CS-B cell lines are defective in the repair of active genes after UV damage, specifically with a lack of repair of the transcribed strand. There is also a slight defect in the repair of the nontranscribed strand. CS proteins have been proposed to play a role in the promoting of access to damage by the repair proteins and more specifically, they may play an essential role in the initiation of transcription after damage is repaired by routing the repair/transcription TFIIH factor, from repair to transcription.

1.1.4 Human NER genes and proteins

Genetic and biochemical analysis

Initial complementation studies that led to the identification of XP, CS and TTD groups were mainly based on analysis of replication repair capability (unscheduled DNA synthesis, UDS) of the different cell lines. Assays employing cell extracts to measure NER have identified defective repair in a number of XP cell lines and have helped to determine overlap between XP cell lines and phenotypically similar rodent repair mutants. Thus, transfection of rodent mutants of which there are 11 complementation groups, with human genomic DNA, has resulted in the identification and molecular cloning of a series of complementing genes designated ERCC (for Excision Repair Cross Complementation) denoting the fact that rodent cell lines were corrected for mutant phenotypes by human DNA. These are called ERCC1, ERCC2, ERCC3, ERCC4, ERCC5, ERCC6 and ERCC8. XP and ERCC lines partially overlap (Wood, 1997a). ERCC2 is equivalent to XP-D, ERCC3 to XP-B, ERCC5 to XP-G, ERCC6 to CS-B, ERCC8 to CS-A and the proteins encoded by ERCC4 and XP-F are equivalent (Reardon et al., 1993) (for more detail see below and Table 1.2). It is noteworthy that most mutants in rodent groups 1 and 4 show extreme sensitivity to DNA crosslinking agents, a feature not identified among human NER mutants. These groups show extreme hypersensitivity (10- to 100-fold) to mitomycin C, cisplatin, melphalan, diepoxybutane, and other agents that produce interstrand crosslinks. High sensitivity to crosslinking agents is not associated with XP or CS cells, but it is one of the hallmarks of cells from Fanconi's anaemia, which have normal NER. Thus, the rodent mutants in groups 1 and 4 are unique amongst the different NER-deficient phenotypes in mammalian cell mutants (Thompson, 1998).

Genes identified using rodent cell mutants

ERCC1

The main focus of this thesis is on ERCC1, the first mammalian DNA repair gene to be cloned by virtue of its ability to complement the UV-sensitive CHO line 43-3B (Westerveld et al., 1984). The gene is approximately 15 kb in size and is located on

human chromosome 19q13.2 where it is closely linked to another human NER gene designated XPD(ERCC2). The ERCC1 cDNA has an open reading frame (ORF) that gives rise to a 1.1 Kb transcript encoding a product of 297 amino acids with a calculated molecular mass of approximately 32.5 kD which contains a nuclear location signal and a putative DNA binding motif. Mutations in the ERCC1 cDNA that are responsible for the phenotype observed in the mutant CHO cell lines 43-3B and UV-4 have been recently identified (Hayashi et al., 1998). Tests of protein-protein interaction, *in vivo* and *in vitro* have demonstrated specific interactions between the human ERCC1 protein and those encoded by the XPA (Park and Sancar, 1994; Nagai et al., 1995; Li et al., 1994a; Li et al., 1995) and ERCC4(XPF) genes (see below) (Biggerstaff et al., 1993; van Vuuren et al., 1993; Park and Sancar, 1994; van Vuuren et al., 1995; Bessho et al., 1997). The human ERCC1 gene was used as a hybridisation probe to isolate the corresponding mouse gene. The mouse and the human genes are highly conserved, although the mouse gene encodes one more amino acid. Overall there is 85% amino acid identity between these two genes. The C-terminus of the human ERCC1 protein is homologous to the C-terminal region of the *E. coli* UvrC protein (Moolenaar et al., 1998) and, also, the translated sequences of the mouse and human ERCC1 genes are homologous with those of the *Saccharomyces cerevisiae* RAD10 gene and the *Saccharomyces pombe* swi10 gene (Rodel et al., 1992), and as mentioned before, expression of Rad 10 protein partially complements ERCC1 deficient cells. The human ERCC1 gene has also been shown to complement the DNA repair deficiency of swi10 mutants of fission yeast (Rodel et al., 1997). RAD10 has been shown to associate with RAD1 in *S. cerevisiae* to form an endonuclease (Tomkinson et al., 1993; Bardwell et al., 1994) that takes part in the mitotic recombination pathway and is required for double-strand break-induced recombination (Ivanov and Haber, 1995), and swi10 associates with the RAD1 homolog RAD16 in *S. pombe* (Carr et al., 1994), where it is thought to form an analogous endonuclease.

Bearing in mind the homology between RAD10 and ERCC1, the latter may also be involved in a recombinational repair mechanism. A puzzling characteristic of ERCC1 comes from phenotypic correction experiments carried out with this gene in DNA repair deficient cell lines. Interestingly enough, when introduced to cell lines from XP groups ERCC1 failed to correct the existing repair defect (van Duin et al., 1988; van

Duin et al., 1989). Nevertheless, the ERCC1 gene product is known to play a critical role in the nucleotide excision repair process as shown by rodent cell lines with a defect in ERCC1 which are hypersensitive to the toxic effects of agents including UV, acetylaminofluorene, 4-nitroquinoline-1-oxide and mitomycin C. This has led to the speculation that mutations in ERCC1 may be developmentally lethal. Alternatively, the hypothesis exists that ERCC1 defects may be associated with a different phenotype, not represented among existing XP or CS groups (McWhir et al., 1993a). As mentioned above, as well as a role in NER, ERCC1 may have other key functions. This is a recurring theme with NER proteins (see XPB/XPD below). The possible involvement of repair proteins in translational and transcriptional control has been proposed as the basis for the neurological and other not obvious NER related problems such as retarded physical and sexual development, as seen in some XP and CS patients (McWhir et al., 1993a).

In the case of ERCC1, there are clues that point towards an involvement of this protein in recombination repair as well as in NER (Sargent et al., 1997) which comes from evidence that ERCC1, and also ERCC4(XPF) mutants, are extremely sensitive to crosslinking agents such as mitomycin C, cisplatin and psoralen (van Duin et al., 1986; Sijbers et al., 1996a; Zdzienicka et al., 1987; Thompson et al., 1994)(see section 1.1.5). However, recent experiments with rodent cells suggest that in mouse, ERCC1 is not essential for the recombination-mediated repair of interstrand crosslinks and that ERCC1 may have an additional role in a process called illegitimate recombination (Melton et al., 1998). In conclusion, the fact that NER proteins seem to have more than one specific role and the different patterns of NER shown by humans and rodents (Mitchell and Hartman, 1990) combine to indicate that the consequences of ERCC1 deficiency are likely to be complex, and may differ between mice and humans.

The ERCC4 (XPF) gene

As already mentioned above, the *S. cerevisiae* ERCC1 homolog, Rad 10 protein, forms a stable and specific complex with Rad1 protein, another component of the NER family of genes. Since human cells possess a RAD10 homolog, it is reasonable to expect that they also have a homolog of the *S. cerevisiae* RAD1 gene. This is indeed the case and the gene is designated ERCC4. This gene has been mapped to human chromosome

16p13.13-p13.2 by somatic cell hybridisation and very recently it has been shown to be the same protein as XPF (Sijbers et al., 1996b). It is interesting to note that there are also indications that the polypeptide encoded by the human ERCC1 and ERCC4 gene interact to form a stable complex in living cells which has been referred to as the ERCC1/XPF complex (Aboussekhra et al., 1995). XPF has been shown to share protein sequence homology with other eukaryotic genes involved in recombination, such as MEI-9 (*Drosophila melanogaster*), and Rad16 (*Schizosaccharomyces pombe*) (Brookman et al., 1996).

The ERCC2(XPD), ERCC3(XPB) and ERCC6(CSB) genes

XPD was cloned using the same basic strategy used to isolate ERCC1 and ERCC4. It is approximately 15 to 20 kb in size, and like ERCC1 is located on human chromosome 19. The gene is organised into 22 exons separated by 23 relatively small introns. Like the ERCC1 translated sequence, that of the XPD gene is homologous with a yeast NER gene, in this case the yeast RAD3 gene. The purified protein has been shown to be a DNA-dependent ATPase with DNA helicase activity whose properties are very similar to those of the yeast Rad3 protein. Defects in XPD gene have been associated with the TTD syndrome (Takayama et al., 1996).

The human XPB gene was cloned by phenotypic complementation of cells from the rodent mutant line RCG3. The gene has been localised to human chromosome 2. The cloned gene is 35-45 kb and contains at least 14 exons. The XPB cDNA has an ORF of 782 codons, which would encode a polypeptide of 89.2 kDa and examination of the amino acid sequence suggests that, like the polypeptide encoded by the XPD gene, the putative XPB polypeptide is a DNA helicase, but with a 3'→5' polarity as opposed to the 5'→3' polarity of the XPD helicase. This has been confirmed by biochemical characterisation of the purified protein. The XPB cDNA complements the UV sensitivity and NER defect in cells from the XP-B genetic complementation group. Interestingly enough, the patient from whom this cell line was established also manifested the clinical features of CS, suggesting that a single gene defect is associated with two clinically distinct hereditary diseases, the implications of which will be dealt with in following sections. Interestingly, the XPB and XPD DNA helicases have also been shown to be components of the p53-mediated apoptosis pathway (Wang et al.,

1996).

The human CSB gene has also been cloned by phenotypic complementation and may be as large as 100 Kb, with at least 21 exons. Its ORF encodes a protein of 108 kD and it is included in the chromosomal region 10q11-21. It also corrects the UV sensitivity of CS-B cells, suggesting that CS group B represents a defect in this gene. Examination of the amino acid sequence of the translated CSB ORF suggests that it too might be a helicase.

The ERCC5(XPG) gene

The cloned gene has an ORF of 1,186 codons and is expected to encode a polypeptide of 133 kDa. ERCC5 protein corrects defective nucleotide repair in extracts of human XPG cells and rodent ERCC5 cells but has no effect when added to extracts of other repair-defective cell lines. Like the yeast Rad1-Rad10 and Rad2 endonucleases, XPG protein cleaves bacteriophage M13 DNA, and it shows a substrate specificity for duplex/3' single strand DNA junctions (O'Donovan et al., 1994) which makes this putative endonuclease an attractive candidate to cut duplex DNA with such a junction at a site of base damage, specifically 3' to the site of damage (Matsunaga et al., 1995). XPG has also been found to bind to PCNA and to share sequence elements with the PCNA-binding regions of FEN-1 and p21 (Gary et al., 1997).

No complementing human genes have been identified for rodent mutants in groups 7, 9 and 10. This is mainly due to the modest sensitivity of these cell lines to UV, which makes the effective selection procedures for isolation of genes a very difficult task.

Genes identified using human cell mutants

As it happens with the nucleotide excision repair-defective bacterial, yeast, and rodent mutants mentioned above, the UV irradiation-sensitive phenotype of XP cells in culture invites a molecular approach to the study of xeroderma pigmentosum through the cloning of XP genes by phenotypic complementation of human cells. Attempts to do so, however, have been largely unsuccessful due to the low stable transfection frequency of human cell lines. The reason for this remains unknown but there are studies pointing to the low efficiency with which DNA sequences are stably integrated and expressed in

human cells compared to rodent cells, that makes the difference even more prominent in those human cell lines specifically defective in cellular responses to DNA damage.

The XPA gene

The human XPA gene is 25 Kb in size and is split into 6 exons. Sequencing the human and murine XPA cDNA showed a single ORF predicted to encode a polypeptide of 273 amino acids with a molecular weight of approximately 31 kD. The XPA gene maps to human chromosome 9q34.1 and there is a 95% homology between the mouse and human sequences at the amino acid level. ERCC1 seems to interact specifically with XPA both *in vivo* and *in vitro* to form a stable complex whose function is explained below (Li et al., 1994a; Park and Sancar, 1994). An initial determination of the domain that mediates this interaction in both ERCC1 and XPA has also been accomplished through amino acid sequencing and structural sequence analysis. XPA is a zinc finger DNA binding protein that has been reported to possess a higher affinity for damaged double stranded DNA than for undamaged double stranded DNA (Robins et al., 1991). Thus, ERCC1 along with the rest of the incision complex may be loaded and possibly oriented to the site of damage by this protein (Nocentini et al., 1997). This model is supported by protein-protein interaction studies showing that it is indeed the case that not only ERCC1 but also the ERCC1-ERCC4(XPF) complex associates with XPA (Park and Sancar, 1994).

The XPC gene

The XPC cDNA has an ORF that can encode a polypeptide of approximately 106 kD, with a basic pI. It shows some homology to the *S. cerevisiae* Rad4 gene, and northern analysis of normal human cells has revealed a single transcript of approximately 3.8 Kb. It has been mapped to human chromosome 3p25. The yeast homolog Rad4 is found complexed with Rad23 as part of a the nucleotide excision repair factor 2 (NEF2) (Schauber et al., 1998; Jansen et al., 1998; Guzder et al., 1998) and has been shown to preferentially bind to damaged DNA (Jansen et al., 1998; Guzder et al., 1998). Similarly, the XPC protein copurifies with the human homolog of Rad23, a 43 kD partner, HHR23B, or sometimes it is found to be bound to a similar protein HHR23A (Sugasawa et al., 1997). XP-C cells are unique amongst XP groups in that they

they manifest only a slight defect in nucleotide excision repair in transcriptionally active genes, but appear to be more severely defective in the repair of transcriptionally silent regions of DNA (Kantor et al., 1990). One implication that can be drawn from this observation is that XPC protein is not required for, and does not participate in, the preferential repair of template strand damage in transcriptionally active genes. In XP-C cells, the low levels of XPC protein may be preferentially recruited to a repairosome complex that participates in the repair of transcriptionally active DNA. This theory, however, has not yet been confirmed (Friedberg et al., 1995). On the other hand, there is a possibility that, in XP-C cells, damage on the transcriptionally active DNA strand is detected by RNA polymerase II (Tornaletti and Hanawalt, 1999). The arrested RNA polymerase would in turn be used as a signal by TFIIH, which would subsequently determine that the lesion to be repaired is in the transcribed strand (Tornaletti and Hanawalt, 1999). In this model, in the non-transcribed DNA, the XPC complex would carry out a similar role. XPC has been termed the ‘initiator’ of global genome repair (Sugasawa et al., 1998). XPC has been shown to preferentially bind to damaged DNA, like its yeast counterpart, and to make a stable footprint at a damaged DNA site (Wood, 1999). Since XPC is one of the NER factors that shows a preference for damaged DNA, it has been proposed that XPC binds tightly to a lesion-distorted region of DNA and alters the DNA structure so the DNA is rendered accessible for the other NER components. If the DNA structure is sufficiently distorted, XPC is dispensable (Wood, 1999).

The CSA(ERCC8) gene

Using functional CSA cDNA isolated from a pEBS7 cDNA library (Thompson, 1998), SV40-transformed CS-A fibroblasts were rescued, which led to the identification of the CSA gene. The CSA sequence maps to human chromosome 5 and encodes a protein that belongs to the WD repeat family of proteins, which appear to have a variety of regulatory functions (Thompson, 1998). The rodent ERCC8 group, which is represented by a mouse lymphoma mutant (US31), corresponds to CSA.

<i>Gene^a</i>	<i>Human disease</i>	<i>Gene, kb</i>	<i>ORF, aa</i>	<i>Chrom.</i>	<i>Protein, kD</i>	<i>Yeast^b equiv.</i>	<i>Properties</i>
ERCC1	?	15	297	19q13.2	33	Rad10p	Endonucleolytic incision (5'-side of damage); complexed with XPF; interaction with XPA; recombination functions?
XPA	XP-A	25	273	9q34.1	31	Rad14p	Damage recognition/DNA binding; zinc finger motif; binding to RPA and ERCC1-XPF complex
XPB/ERCC3	XPB-CS	45	782	2q21	89	Rad25p/ Ssl2p	3'→5' helicase; member of TFIIH complex; essential for transcription initiation; formation of preincision complex
XPC	XP-C	24	940	3p25.1	106	Rad4p	Specific to global repair pathway; complexed with HHR23B; binding to ssDNA; stabilisation of preincision complex
XPB/ERCC2	XP-D, TTD, XPD-CS	19	760	19q13.2	87	Rad3p	5'→3' helicase; member of TFIIH complex; essential for transcription initiation; formation of preincision complex
DDB1	XP-E?	?	1140	11q12-13	127	?	Recognition/DNA binding of UV photoproducts and some bulky lesions; absent in some XP-E lines
XPF/ERCC4	XP-F	30	916	16p13.13	104	Rad1p	Endonucleolytic incision (5'-side of damage); complexed with ERCC1; interaction with XPA, recombination functions?
XPG/ERCC5	XP-G, XPG-CS	32	1186	13q33	133	Rad2p	Endonucleolytic incision (3'-side of damage); junction-specific nuclease; interaction with XPC?
CSA/ERCC8	CS-A	?	396	5	44	Snf2p	Preferential repair of active genes; repair of transcribed strand; transcriptional regulator?
CSB/ERCC6	CS-B	~85	1493	10q11.2	168	Rad26p	Preferential repair of active genes; repair of transcribed strand; transcriptional regulator?
HHR23A	?	?	363	19p13.2	40	Rad23p	Ubiquitin-like N-terminus; identified by homology with HHR23B, a homolog of RAD23
HHR23B	?	?	409	3p25.1	43	Rad23p	Specific to global repair pathway; complexed with XPC; ubiquitin-like N-terminus
p62TFIIH	?	?	548	11p14	62	Tfb1p	Component of TFIIH; essential for viability
p44TFIIH	?	?	395	5q1.3	44	Ssl1p	Component of TFIIH; essential for viability; TFIIA-like zinc finger motif
p52TFIIH	?	7	436	6p21.3	52	Tfb2p	Component of TFIIH; essential for viability; TFIIA-like zinc finger motif
LIG1	46BR	53	919	19q13.2	102	Cdc9p	Nick closure in the final step of repair

Table 1.2: Cloned human nucleotide excision repair genes and their encoded proteins. In addition to the proteins listed, the complete excision repair reaction requires trimeric RPA, additional components of the TFIIH complex, polymerase accessory factors RFC, PCNA, and polymerase δ/ϵ (Nichols and Sancar, 1992; He and Ingles, 1997). The data on p52TFIIH was taken from Marinoni, 1997. (For more detail on these proteins refer to the Replication section below).

^aBy agreement the name XPB has replaced XPBC and ERCC3, and so forth, but ERCC names can be preferable in certain contexts.

^b*S.cerevisiae*

This Table has been adapted from Thompson, 1998.

1.1.5 NER in mammalian cells: Biochemical mechanisms

Knowledge of the actual mechanisms and protein functions in the NER pathway³ is mostly based on *in vitro* studies involving both recombinant proteins and purified proteins obtained by chromatographic methods from cells or tissues (Aboussekhra et al., 1995). Figure 1.2 presents an outline of the steps involved in the nucleotide excision repair mechanism in global DNA. Although the NER reactions presented in this Figure are outlined as a series of individual steps, these reactions may well be concerted and form a continuum where there is no need for the formation of stable intermediates (Thompson, 1998). Excinuclease is a term that has been introduced to refer to the excision nuclease activity that cuts on both sides of the damaged site.

Damage recognition

The fact that XP-A patients present a very severe form of XP and that XP-A cell lines are highly UV sensitive, suggests that XPA plays a key role in NER by recognising the damaged site on the DNA. Support for this comes from experiments that have shown that XPA presents higher affinity for UV-irradiated DNA than for undamaged DNA (Thompson, 1998). It also presents higher affinity for single-stranded undamaged DNA than for double-stranded undamaged DNA. Its sequence has a zinc finger motif and in cell-extract assays, no repair incision is observed in the absence of XPA. Different mutations in XPA may also have subtle effects on damage recognition.

Replication protein A (RPA) is also involved in damage recognition (Sancar, 1994). RPA is a heteromeric complex that comprises the subunits p70, p32 and p14 (also known as HSSB). RPA is essential for the incision step of the repair reaction and in addition it has been shown to be required for the replication of the SV40 chromosome *in vitro*. It binds to ssDNA through the p70 and p32 subunits and also binds to XPA in a cooperative fashion (Saijo et al., 1996).

XPA also binds specifically to ERCC1 (Li et al., 1994a; Park and Sancar, 1994) and the regions of XPA that are necessary for this interaction have been mapped (Li et al.,

³ The data presented in this section regarding NER biochemical mechanisms has been taken from Thompson, 1998 and Wood, 1997. Additional comprehensive reviews can be found in Sancar, 1994.

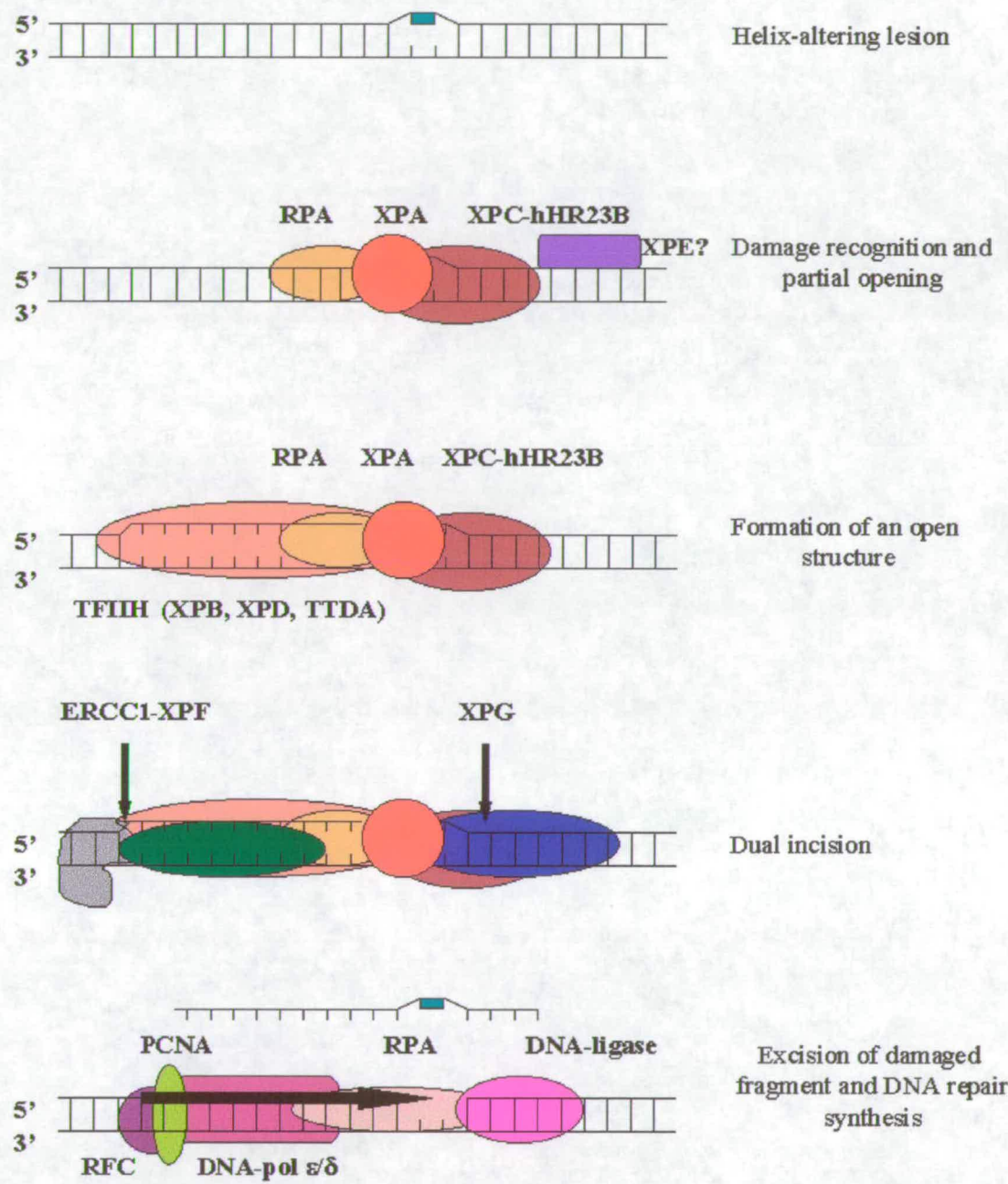


Figure 1.2: Model of NER in the global DNA repair pathway. The roles of XPE and XPC-HHR23B complex are not well understood. Adapted from Wood, 1997.

1995). It has been proposed that this interaction may also enhance damage recognition (Nagai et al., 1995). In addition, XPA interacts with the TFIIH factor. All these interactions suggest that XPA plays a key role recruiting NER proteins.

DNA damage binding protein (DDB) or XPE binding factor (XPE-BF) is another protein that seems to be required for the recognition step, but its specific role remains unknown (Thompson, 1998).

Mechanism of open complex and dual incision formation

The mechanism of open complex and dual incision formation has been studied in detail using *in vitro* cell free extracts (Mu et al., 1996; Evans et al., 1997). Additional information has been recently derived from experiments using electrophoretic mobility assays and DNase I footprinting assays (Wakasugi and Sancar, 1998). XPB and/or XPD helicases seem to be involved in the unwinding of the DNA duplex before incision. XPD unwinds the DNA in a 5' to 3' direction and XPB unwinds in the opposite direction. Both proteins are subunits of the basal transcription factor TFIIH, which is required to initiate transcription of most RNA polymerase II class genes (see below for implications of this fact in human NER syndromes). As mentioned before, XPA interacts with TFIIH, which suggests that TFIIH is recruited to the site of damage by XPA with the possible help of RPA which would stabilise the open DNA complex until the incision step takes place.

The mechanism of lesion removal in mammalian cells involves endonucleolytic incision on both sides of the damaged DNA as in *E.coli* but with a much larger distance between the lesion and the 5' incision site (Evans et al., 1997). The excised fragment is approximately 27-29 nucleotides long which corresponds to one incision at the third to fifth phosphodiester bond on the 3'-side of the lesion and a second incision at the 22nd to 25th phosphodiester bond on the 5'-side. Each incision is carried out by a different nuclease activity (see Fig. 1.2).

The 3'-side incision

XPG is a single-strand specific endonuclease that acts on the 3'-side of the lesion (O'Donovan et al., 1994) and precedes the 5'-side incision. XPG has homology with the

human FEN1 (flap endonuclease), which cleaves the 5'-single-stranded end at a single-strand to double-strand junction. Targeting of XPG to the lesion may be mediated by RPA which is required for both the 3' and the 5' incision steps (Thompson, 1998).

The 5'-side incision

This step is carried out by a complex composed of ERCC1 and XPF (Park et al., 1995; Matsunaga et al., 1995). This complex is necessary and sufficient to carry out this incision *in vitro*. The purified ERCC1-XPF complex has a preference for single-stranded DNA and the single-stranded part of a DNA duplex containing a bubble region (deLaat et al., 1998). Anti-ERCC1 antibodies inhibit the 5'-incision specifically, resulting in uncoupled 3'-incision in a defined system. The ERCC1-XPF complex is analogous to the yeast (*S. cerevisiae*) Rad1-Rad10 endonuclease complex (Tomkinson et al., 1993), which cuts specifically at the junction of a Y-shaped DNA and only in the strand with the 3'-single-stranded terminus (Bardwell et al., 1994). Targeting of the ERCC1-XPF complex is likely to occur through the action of XPA. RPA may confer structure-specific incision activity to both ERCC1-XPF and XPG (Thompson, 1998).

Role(s) of XPC-HHR23B

The exact role(s) of the XPC-HHR23B complex is still unknown. Although XPC is normally found complexed to HHR23B *in vivo*, in the highly purified, reconstituted excision nuclease assay, there is no requirement for HHR23B. XPC is required for *in vitro* incision of substrates and for transcription coupled repair *in vivo*. The complex may help to stabilise the open DNA structure at the 3'-side of the damage and help target the incision nuclease activities to ensure appropriate specificity. Recently, XPC has been proposed to be the 'initiator' of global genome repair (Sugasawa et al., 1998) (see section 1.1.4).

Excision of damaged segment

In vitro analysis seems to indicate that the excised oligomer (still bound to protein) is released from the DNA duplex by the incision nuclease machinery without assistance from the repair synthesis proteins.

Synthesis and ligation

PCNA and RFC

The DNA polymerase accessory factor, proliferating cell nuclear antigen (PCNA, see Replication section 1.2 for further information on PCNA in the context of replication), is required *in vitro* for the repair synthesis step, but not for the incision step (see Fig. 1.2). PCNA increases excision efficiency and it has also been shown that it may be necessary for repair synthesis *in vivo* (Li et al., 1996; Miura et al., 1996). These observations have led to the implication of DNA polymerase- δ or - ϵ in the polymerisation step of repair (see Replication section 1.2 for more information of pol- δ and - ϵ). The polymerase accessory factors PCNA and RFC may be required to displace the incision proteins from the postincision complex. The trimeric PCNA protein, which acts as a clamp to increase the polymerase processivity, requires RFC (a multimeric complex consisting of 5 subunits) for its loading onto the DNA.

DNA ligase

The final step of the repair process requires the action of a DNA ligase. Four different DNA ligases have been identified but their roles are still under characterisation. DNA ligase I participates in DNA replication and evidence for its involvement in excision repair comes from a single human cell line, 46BR, isolated from a patient with a complex syndrome, including photosensitivity (Webster et al., 1992). 46BR cells, which have mutations affecting both alleles of the *LIG1* gene, are somewhat UV-sensitive and show an abnormally high level of incision-associated breaks after UV treatment.

Functional heterogeneity of NER proteins

The nucleotide excision repair/transcription relationship⁴

At this point it would be interesting to note how XPB and XPD proteins also form part of the machinery involved in RNA polymerase II-dependent transcription (Drapkin et al., 1994). Protein immunoprecipitation and correction of defective phenotype assays indicate that XPD protein is an integral component of human factor TFIIH (the

⁴ For a review see Friedberg, 1996; Hanawalt, 1994.

multiprotein complex required for the initiation of basal transcription by RNA polymerase II) and is required for both transcription and NER (van Vuuren et al., 1994; Hanawalt, 1994). What is more, purified preparations of TFIIH have been shown to contain the NER protein XPB(ERCC3) (Schaeffer et al., 1993). As mentioned before, TFIIH has another three components, p62 and p44, p34 (Humbert et al., 1994) and a fourth, p52 (Marinoni et al., 1997). The significance of this bifunctionality is demonstrated by the fact that it is also preserved in yeast. What the exact implications of such a characteristic are is something that still remains to be seen, but the fact that these proteins are involved in both repair of DNA and transcription-related processes opens up a broad range of highly interesting questions about the role they may play in the pathology of syndromes such as XP, CS or TTD where complications not directly related to DNA repair deficiency, i.e., mental retardation and impaired sexual development, are seen (Prakash et al., 1996). It also raises the logical question of whether other proteins already identified as being part of the NER machinery and whose properties up to now seem to point towards more complicated functions than that of DNA repair alone could also have a prominent role in transcriptional control and other DNA metabolism processes.

*The nucleotide excision repair/recombination repair relationship?*⁵

As already mentioned, ERCC1, and also ERCC4(XPF) mutants, are extremely sensitive to crosslinking agents such as mitomycin C, cisplatin and psoralen which suggests that these mutant cell lines are defective in the repair of interstrand crosslinks.

It is known that in *E. coli* the repair of interstrand crosslinks occurs in three basic steps: dual incisions of one strand by (A)BC endonuclease, homologous pairing with a sister duplex mediated by RecA, and finally dual incisions in the second strand by (A)BC endonuclease. Interstrand crosslinks are also removed from mammalian cells but there are no details on the mechanism(s) that repair this type of damage (see Fig. 1.3). Based on mutagenesis studies, it has been hypothesised that crosslinks are removed from human cells by two mechanisms. In error-prone repair, following the dual incisions a DNA pol fills in the gap by passing the lesion to generate a three-

⁵ For a report of recent advances on this subject see Recent advances in DNA repair and recombination (Iwanejko and Jones, 1998).

strand intermediate that is then repaired like any other endonuclease substrate. In the error-free mechanism, dual incisions on one strand are followed by strand transfer from the intact duplex and ligation, and dual incisions of the second strand are followed by release of the crosslink and repair synthesis, in a manner similar to crosslink repair in *E. coli* (see Fig. 1.3). The exact role played by ERCC1 in the recombination repair mechanism, if any, is not known but the fact that its yeast homolog, RAD10 along with the yeast XPF homolog, RAD1, has been shown to take part in mitotic recombination (Bardwell et al., 1993; Bardwell et al., 1994), seems to support the idea that ERCC1 is likely to be involved in this type of repair.

More recent evidence derived from experiments carried out by Melton and co-workers (1998) suggests that although ERCC1 is likely to be involved in homologous recombination it is not an essential component of that repair mechanism. Melton *et al.* propose that ERCC1 is also involved in a process called illegitimate recombination, which is used by rodents to prevent the accumulation of double strand breaks adjacent to unrepaired lesions on replicating DNA (Bierne and Michel, 1994).

The ERCC1 paradox

Mice homozygous for ERCC1 disruption, which has no counterpart among human disorders, showed unexpected pathologies, including runting at birth (see Fig. 1.4), early death, liver aneuploidy, and elevated p53 levels in liver, brain and kidney (McWhir et al., 1993a), possibly due to the lack of repair of spontaneous oxidative damage to DNA. On the other hand, mice carrying disruption mutations in the XPA and XPC loci were found to mimic XP patients in their pathology. They showed greatly increased susceptibility to UV radiation- and chemical carcinogen-induced skin tumours (deVries et al., 1995; Nakane et al., 1995), along with other skin and eye lesions consistent with the human disease. XPA-/- transgenic mice appeared physiologically normal at birth and did not develop (by 18 months) the nervous system pathology seen in XP patients. In addition, a mouse model has also been developed that carries a point mutation in the XPD gene and mimics the TTD syndrome. TTD mice present brittle hair, developmental abnormalities, reduced life span, UV sensitivity, and skin abnormalities (de Boer et al., 1998).

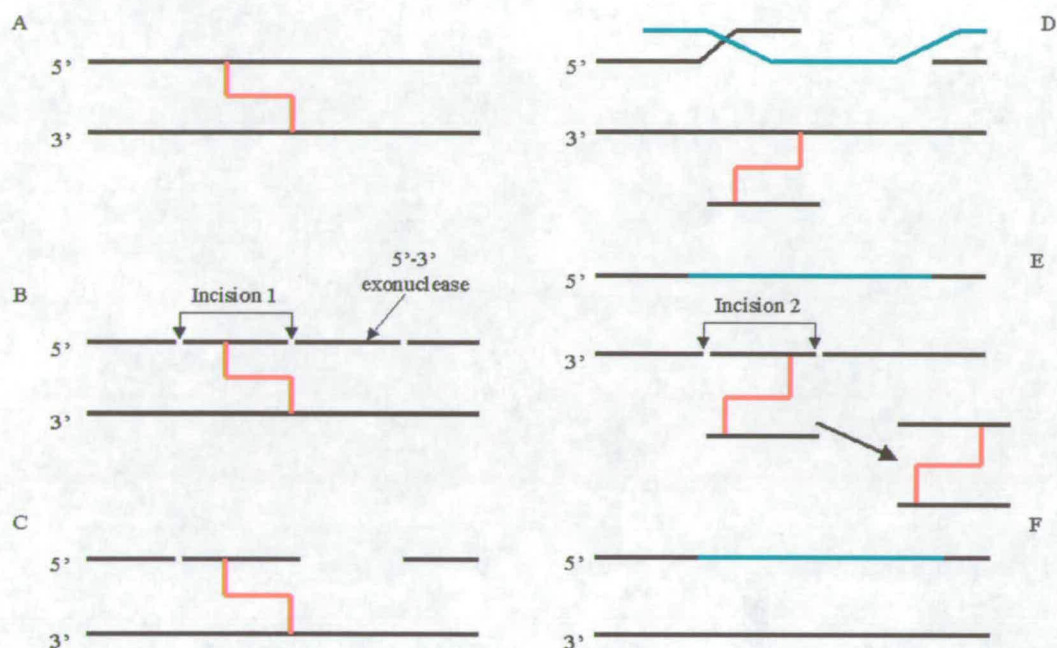


Figure 1.3: Model for the repair of DNA interstrand crosslinks. (A) Cross-linked DNA. (B) The UvrABC endonuclease cuts the DNA generating incisions which flank the cross-link (incision 1). (C) It is proposed that the 5'-3' exonuclease of Pol I then generates a gap 3' to the site of the cross-link, which is a substrate for RecA-mediated recombination. (D) During recombinational repair of the gap, an invading homologous DNA strand displaces the cross-linked oligonucleotide. (E) Once recombination is completed, the second DNA strand is incised by the UvrABC endonuclease (incision 2), leading to the excision of a complex 11-mer or 12-mer oligonucleotide structure. (F) DNA repair is completed by repair synthesis and ligation. Taken from Friedberg, 1995.

The less severe effect of XPA mutations compared with ERCC1 mutations in the mice suggests an involvement of the ERCC1 gene in some critical process during development other than NER, perhaps a role in recombination repair or cell cycle control (control of replication and mitosis, see next sections). This thesis tries to investigate these possibilities.

Premature ageing syndromes: A link with DNA repair processes?

There are several rare conditions in man that are associated with premature ageing. The most widely studied progeroid syndromes, as these conditions are known, are

A



B

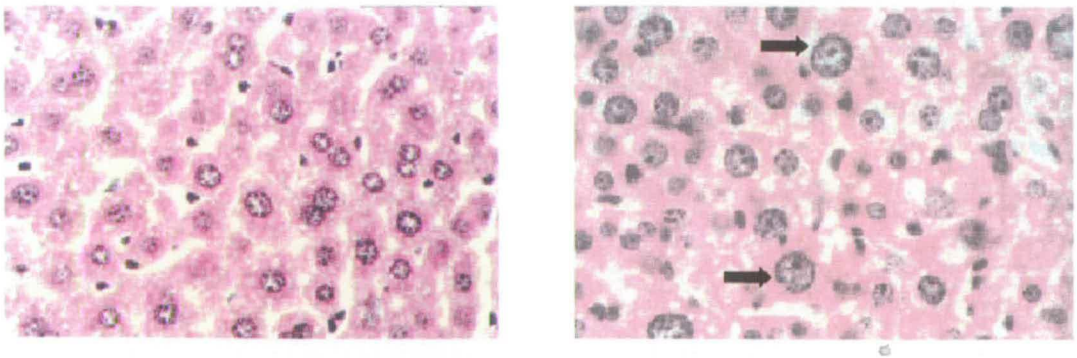


Figure 1.4: A) Photograph of a ~20 day-old wild type mouse and its ERCC1-deficient littermate showing the runted appearance of the transgenic ERCC1-/- mouse. B) Haematoxylin-eosin liver sections from ~20 day-old ERCC1 -/- mice; 40x magnification. Left panel: wild type liver section (note homogeneous distribution of small nuclei); right panel: ERCC1-/- liver section showing some small nuclei (normal) and a number of enlarged aneuploid and polyploid hepatocyte nuclei (black arrows).

Hutchinson–Gilford progeria, Werner’s syndrome and Cockayne’s syndrome, but the groups also includes Bloom’s syndrome, ataxia telangiectasia and Down’s syndrome.

Hutchinson–Gilford progeria is a rare genetic disease with a birth incidence of 1 in 8 million and caused by a mutation of unknown origin (see Chapter 6 for more details). Werner’s syndrome is an autosomal recessive disorder with a prevalence rate of 1 in 10 million people. Unlike progeria, where the phenotype of the patients is apparent in early infancy, Werner’s syndrome is not apparent until the third or fourth decade (progeria of the adult). Cockayne’s syndrome has already been described elsewhere in the previous sections in the context of NER–related syndromes. Bloom’s syndrome is characterised by severe pre– and postnatal growth deficiency, a sun–sensitive erythematous face, hyper– or hypo–pigmented skin lesions and variable immunodeficiency. Ataxia telangiectasia (AT; see Replication section 1.2 and Chapter 4) and Bloom’s syndrome have been extensively studied to establish the genetic basis of various cancers. A summary of the most prominent characteristics of these syndromes is presented in Table 1.3.

	<i>Progeria</i>	<i>Werner’s</i>	<i>Cockayne’s</i>	<i>Bloom’s</i>	<i>AT</i>
Onset	Infancy	3rd decade	2nd year	Infancy	Infancy
Death (decade)	2nd	5th/6th	2nd	2nd/3rd	Childhood
Stunted growth	present	present	present	present	present
Abnormalities					
Skin	present	present	present	present	absent
Skeletal	present	present	present	present	absent
Muscular	present	present	present	absent	absent
Visual impairment	absent	present	present	absent	n.r. ¹
Mental deficiency	absent	absent	present	n.r.	n.r.
Photosensitivity	absent ²	absent	present	present	present
Cancer risk	absent	present	absent	present	present

Table 1.3: Characteristics of some premature ageing syndromes. ¹n.r. stands for not reported; ² Although patients suffering from progeria do not present photosensitivity there are reports that cells derived from these patients present defects in repair synthesis and show abnormalities in their response to UV damage (Lipman et al., 1989; Wang et al., 1990; Wang et al., 1991). Adapted from Dyer and Sinclair, 1998.

Cockayne’s syndrome has already been mentioned in the context of DNA repair since cells derived from CS patients show impaired DNA repair ability and hypersensitivity to UV (see above). Defects in the XPB and XPD helicases have been identified as being

the cause of the phenotype shown by some of the cell lines derived from XP-CS patients (see above and Table 1.2). Bloom's syndrome cells present chromosomal instability and chromosomal breaks are a common feature of these cells. BS patients are homozygous for defects in encoding the BLM gene on the long arm of chromosome 15 which has been identified as being a helicase (Ellis et al., 1995). In addition, the gene responsible for Werner's syndrome has also been identified as a helicase on chromosome 8 (Yu et al., 1996). Furthermore, cells from ataxia telangiectasia patients show spontaneous chromosomal instability and are hypersensitive to the effects of ionizing radiation. Overall, it seems that these syndromes support the idea that ageing and DNA repair are intimately related (see Chapter 6 for more information) in that ageing may result from an imbalance between DNA damage, DNA damage responses (see Replication on damaged DNA template, section 1.3 and Cell Cycle Control, sections 1.5 and 1.6 for further information on this topic) and repair.

1.2 DNA Replication: A Mechanistic Overview

The complementarity of the two DNA strands first suggested by Watson and Crick was an obvious clue as to how DNA replicates. All replication processes simply involve the melting apart of those two strands followed by the polymerisation of each complementary strand on the resulting single-stranded templates. However, in many cases, the complexity of the enzymatic machinery involved in DNA replication is greater than would have been expected, given that the information necessary to encode two daughter genomes is encoded in the parental genome in such a simple way. This complexity has presumably evolved to increase the efficiency and fidelity of DNA replication and to ensure that the process of DNA replication is coordinated with other events in the life of a cell. Below I will try to lay out the basics of the DNA synthesis process paying special attention to the relationship between DNA replication⁶ and the repair of DNA damage. A summary of the replication proteins known to date is presented below and in Table 1.4.

⁶ For a complete review of the subject it is recommended to read *DNA Replication in Eukaryotic Cells* (DePamphilis, 1996) and references therein.

<i>Protein</i>	<i>Replicative Function</i>
DNA polymerases	
pol- α :primase	DNA polymerase, primase
pol- δ	DNA polymerase, 3' to 5' exonuclease
pol- ϵ	DNA polymerase, 3' to 5' exonuclease
Accessory proteins	
RPA	single-stranded DNA binding
PCNA	pol- δ and pol- ϵ processivity factor
RFC	loads PCNA onto template
Nucleases	
ribonuclease H1	Okazaki fragment maturation
FEN-1(MF-1)	Okazaki fragment maturation
Others	
DNA ligase I	joins Okazaki fragments
topoisomerase I	unlinks parental strands
topoisomerase II	unlinks parental strands and progeny duplexes

Table 1.4: Cellular replication proteins. Adapted from Brush, 1996

1.2.1 Protein-protein interactions at the replication fork

The viral T antigen, pol- α :primase and RP-A are required for the initiation of DNA replication in the SV40 system. Several studies have provided evidence for the interactions among these three proteins (for references see Brush and Kelly, 1996) and these interactions are likely to be important factors that contribute to the efficiency and specificity of the initiation reaction. Unwinding of the SV40 origin requires both T antigen and RP-A. Once the DNA is unwound, the interaction between pol- α and T antigen is likely to play a role in the efficient priming on the RP-A-covered, single-stranded template. Priming is also likely to be influenced by an RP-A/primase interaction, which has been shown to occur *in vitro*. Confirmation of similar interactions in mammalian origins of replication awaits the identification of the cellular counterpart(s) of T antigen.

Protein-protein interactions also play an important role in the elongation phase of DNA replication. For example, the processivity of pol- δ is absolutely dependent on its interaction with PCNA, which is topologically linked to the template DNA. There is no evidence to show interaction between the polymerases pol- δ and pol- ϵ and other replication proteins.

Molecular interactions among proteins may also be important in the final stages of DNA replication, when the Okazaki fragments are joined to form a continuous DNA chain. The specific requirement for DNA ligase I in the ligation of the resulting Okazaki fragments may reflect the strict requirement for specific contacts between this protein and other proteins involved in the maturation of daughter strands.

Moreover, interactions between replication proteins and other cellular factors have also been described. These interactions are likely to play a role in the regulation of DNA replication, although their functions are not totally clear. For instance, RP-A is known to interact with the transcription factors p53, GAL4 and VP16 and the repair proteins XPA and XPG (for references see Brush and Kelly, 1996). In fact, RP-A is required for *in vitro* Nucleotide Excision Repair (NER) where it takes part during DNA repair synthesis (Friedberg and Wood, 1996). Additionally RP-A is required for damage specific incision (see Friedberg and Wood, 1996 and references therein). The yeast homolog of the heterotrimeric protein RP-A is encoded by the RFA genes. Mutations in the RFA1 gene of *S. cerevisiae* result in yeast strains abnormally sensitive to UV, which suggests that RP-A may also have a role in NER *in vivo*.

PCNA is also required for the repair synthesis step of NER in mammalian cells *in vitro*, and there is also evidence that PCNA is involved *in vivo* (see Friedberg and Wood, 1996 and references therein). These observations and data obtained from experiments using chemical inhibitors, implicate pol- δ or pol- ϵ in the repair synthesis step. DNA pol- ϵ has been shown to be active in NER *in vitro*, where the presence of RP-A and replication factor C (RFC) is also required. As shown in Table 1.4, RFC seems to be required to load PCNA onto the DNA template.

More recent evidence comes from studies of the anatomy of the DNA replication fork achieved by reconstitution of SV40 DNA replication *in vitro* (Waga et al., 1994). In this refined model it has been shown that DNA pol- α :primase functions primarily to synthesise RNA-DNA primers for initiation of DNA replication at the origin and for priming each Okazaki fragment. A polymerase switching reaction which requires replication factor C and PCNA allow two molecules of DNA pol- δ to replicate both strands of the double helix at the same time (Waga et al., 1994).

In summary, there seems to be a number of essential protein–protein interactions among the replication proteins themselves and also among the replication proteins and proteins that are part of the NER machinery (XPA, XPG) or that function as regulators of the cell cycle (p53). Also, some of the replication proteins play an active role in the NER process itself (RP–A, RFC, PCNA). This suggests that these two cellular functions are intimately related and below special attention will be paid to this fact.

1.3 Effects of DNA damage on DNA Replication

When DNA is damaged by UV irradiation, ionizing radiation or chemicals, it must be repaired to prevent genomic alterations that could otherwise give rise to mutation, death or the appearance of cancer cells. In order to avoid this, cells have evolved a number of mechanisms to repair their DNA, and to prevent DNA replication until the damage is repaired (Lu and Lane, 1993)(see next section).

Somewhat paradoxically, however, the number of lesions that a cell can tolerate without significantly losing its capacity to reproduce is surprising. In fact, quite often lesions are not eliminated before the portion of the genome where they are located is replicated or transcribed. For instance, XP human fibroblasts are defective in NER and therefore can not repair the damage caused by UV. However, 37% of these cells manage to survive after UV doses that cause considerable damage to their DNA (approx. 16000 dimers per genome) (Meneghini et al., 1981).

It is reasonable to assume that the replication apparatus can not proceed normally through a DNA structure that contains a helix–distorting factor (such as the pyrimidine dimers caused by the UV), but somehow the cell manages to deal with these and other lesions during replication. What are the biological implications of DNA replication on a damaged template? How does a cell react to DNA damage in terms of replication? What are the mechanisms that cells use to tolerate DNA lesions? What replicative behaviour would be expected after DNA damage from cells that can not repair DNA? In this section special attention will be paid to provide an answer for these questions⁷.

⁷ For an extensive, comprehensive review on this subject see Meneghini *et al.*, 1981. Also see Friedberg et al., 1995, Chapters 10–12

1.3.1 The biological effects of DNA lesions

The most extensively studied mammalian system to investigate the biological consequences of DNA lesions has been the Xeroderma Pigmentosum (XP) cell strains. As mentioned before (DNA Repair section 1.1), these cell lines are fibroblasts derived from patients with the disease xeroderma pigmentosum and are characterised by a defect in the repair of some DNA lesions (mainly pyrimidine dimers). These cells are particularly useful to compare their response to DNA damage with that of wild type cells.

Effects on cell killing

Using a method for the measurement of the disappearance of pyrimidine dimers after UV irradiation and correlating this with the survival after different doses of UV, it was possible to see that NER proficient cells eliminate 80% of the dimers in 2 days whereas the XP cells (XP-A complementation group), although originally having fewer dimers, eliminate only a very small fraction during a 4-day period. The dose of UV light that results in a survival of 37% of the cell population in wild type fibroblasts is 2.6 J/m^2 . For XP cells it is 0.4 J/m^2 (Haseltine et al., 1980). This dose is still capable of producing a high number of dimers per genome. Since the XP cells can not repair this damage and still 37% of them manage to survive, it is likely that lesions must somehow be tolerated without significantly affecting the survival of the cell. However, the overall consequence of the persistence of dimers in the genome is the reduced survival of the XP cells compared to the repair-proficient controls, for the same doses of UV light. These two statements do not necessarily contradict each other. If we assume that a certain number of dimers correspond to a lethal event, it is logical to think that the burden of dimers that gives one lethal event per cell in a repair proficient cell population is bigger than that in an XP cell population, since in the wild type cell population most of those dimers will be eliminated by the excision repair mechanism.

Other experiments performed under conditions that allow cells to repair their DNA before undergoing replication (by synchronising them in a pre-replicative state) also show that DNA lesions have a deleterious effect on cell survival. Under these conditions, when DNA repair proficient cells are irradiated in confluence (this arrests the

cells at a pre-replicative state) and released after various lengths of time, they show a gradual increase in survival (Meneghini et al., 1981). On the other hand, XP cells show no or little survival increase under the same conditions.

Cell mutation

Mutations are another effect of DNA lesions. Proof of this also comes from the comparison between normal human and XP cells. At the same UV dose, the frequency of mutations induced by UV light is higher in XP cells than in normal cells. Nevertheless, at equitoxic UV doses, the mutation frequency in XP cells is the same as that induced in normal cells. An equivalent reason to the one given above applies here. In normal cells, where DNA lesions are repaired, the number of dimers required for a lethal event to occur is much higher, but probably these cells end up with the same lesion burden as the XP cells at the time the mutation event takes place. It is interesting to note that fibroblasts derived from patients with an XP variant (these cells show the same excision capability as normal human cells) nevertheless shows a higher mutation rate, even at equitoxic doses (Maher et al., 1976).

When cells are subjected to the liquid holding technique, (i.e., irradiated while synchronised in a pre-replicative state so that time for repair before entering replication can be controlled by controlling the time at which the cells are released post-irradiation), normal human fibroblasts prevented from replicating for several days show background (unirradiated) mutation levels, whereas XP cells continue to have the same high mutation frequency and do not show a reduction with time (Maher et al., 1979).

All this evidence tends points towards a role for pyrimidine dimers in the production of mutations (for a review on this topic refer to Friedberg et al., 1995, Chapter 12).

Cell transformation

XP and ataxia telangiectasia patients have provided clues towards a direct link between unexcised lesions and cancer. These patients are characterised by having an increased incidence of specific types of cancers (skin cancers mainly) and their cells lack the ability to repair certain type of lesions (for more detail refer to the previous section: DNA Repair).

However, human cells are transformed with difficulty *in vitro* so other systems such as the more easily transformed mouse 3T3 cells have been used to test the relationship between unexcised DNA lesions and cell transformation. 3T3 cells have been subjected to the liquid holding technique and assayed for transformation. If after exposure to the damaging agent 4-nitroquinoline-1-oxide (4NQO) these cells are allowed to repair their DNA in a non-replicative state, a decrease in the frequency of oncogenic transformation occurs and this decrease has been shown to be directly related to the rate of enzymatic removal of the 4NQO-induced adducts from the DNA of the cells (Meneghini et al., 1981). This seems to indicate that in fact, the accumulation of DNA lesions leads to the transformation of these cells.

These experiments have not been carried out with the mouse XP-equivalent cells, but this a control experiment that needs to be carried out. If unexcised DNA lesions are indeed responsible for transformation, these cells, when subjected to the liquid holding technique, would maintain their frequency of transformation even after long periods in the pre-replicative state.

Sister chromatid exchanges

The biological significance of sister chromatid exchanges (SCE) comes through its possible relationship to mutagenesis and carcinogenesis. SCE consists of the exchange of homologous parts of sister chromatids in mitotic cells. SCE is a phenomenon that involves exchanges of double-strand DNA and several mutagenic and carcinogenic agents are known to enhance it in mammalian cells.

The fact that UV-irradiated cells pass through a period of DNA synthesis before SCEs are produced seems to point towards a role for unexcised lesions in the formation of SCEs (Meneghini et al., 1981). Regarding this point it is important to know what happens to the replication process/machinery when a lesion in the DNA is reached. Does the cell bypass the arrest caused by the lesion? From the evidence discussed in this section this appears to be the case, but how does the cell manage to do this? Is this process faithful or structural alterations in the DNA arise with it that in the end lead to several biological end-points? These questions are addressed in the following section.

1.4 Replication on a Damaged Template

The study of this particular aspect of the cell biology is not easy due to a number of reasons. Firstly, the normal replication process in mammals is very complicated and the knowledge about it is still poor. Secondly, different mammalian systems may have different ways of dealing with the problem and it is difficult and risky to extrapolate from one system to another. Thirdly, the experimental methodology that has been used to elucidate this problem, namely sucrose–gradient centrifugation, is subjected to methodology variations which makes the standardisation of the conclusions derived from the data difficult.

1.4.1 Replication on a damaged template in bacterial systems and lower eukaryotes

In order to introduce the subject, I will discuss the processes of replication on damaged templates in bacterial systems. The knowledge of these mechanisms is more complete in bacterial systems and it will serve to introduce some concepts that will later be used in the discussion of the mammalian process.

When the replication machinery reaches a pyrimidine dimer, synthesis of DNA is blocked at that point and replication resumes at a point beyond it, resulting in the creation of a gap opposite the lesion (Rupp and Howard-Flanders, 1968) (see Fig. 1.5). Although there is no conclusive proof that the gap is located opposite the dimer, there is some evidence that points towards this fact. For example the size of the daughter strand synthesised after UV irradiation, as measured by alkaline sucrose gradients, is approximately the same as the interdimer distance (Rupp and Howard-Flanders, 1968). The repair of the gap in bacterial systems is mainly carried out by a single strand exchange mechanism which fills the gap with undamaged DNA from the opposite isopolar parental strand. This process requires the Rec A and Lex A genes, amongst others (Friedberg et al., 1995). This recombination process provides the bacteria with a way to avoid replication of the damaged region, but it creates a gap in the parental strand. This gap is however easily filled in by repair synthesis (Meneghini et al., 1981). This mechanism has been termed both ‘post–replication repair’ and ‘recombinational repair’

(see Fig. 1.6).

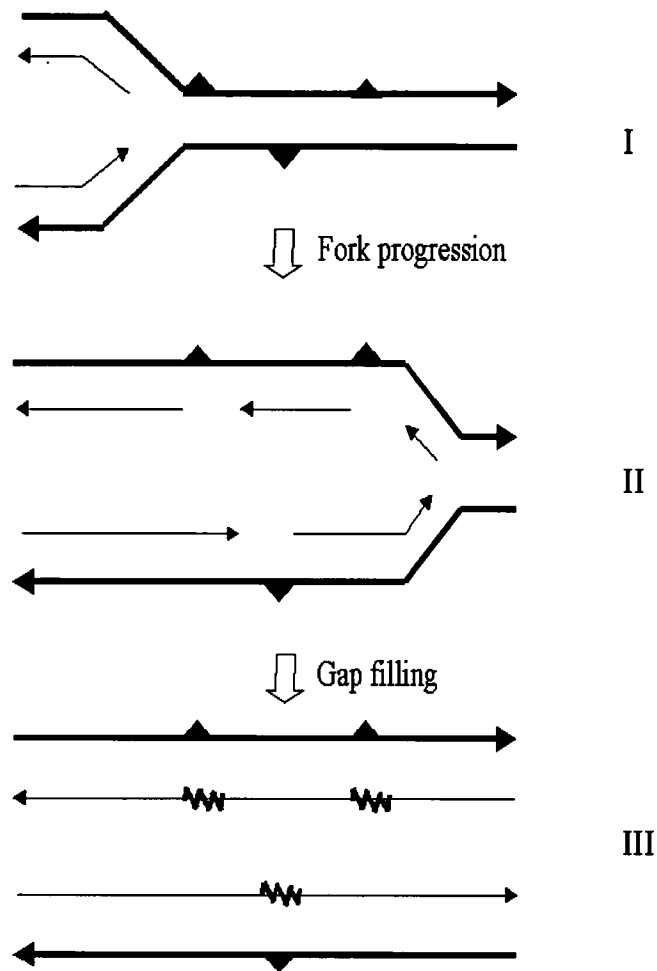


Figure 1.5: Schematic model of the mechanism of gapped DNA synthesis. When the growing fork reaches a lesion (represented by the solid triangles) in the parental strand, replication of the daughter strand terminates (I). Replication reinitiates further along the template. This leaves gaps in the daughter strand (II) which are subsequently filled by *de novo* synthesis or recombination (see Fig. 1.6)(III). Adapted from Doniger, 1978.

Bacterial cells, in addition, seem to have another way of dealing with unexcised lesion during replication. This is the process called 'translesion synthesis' where a 3'-5' editing exonuclease activity associated with the bacterial DNA polymerase is responsible for the block in DNA synthesis at the lesion. The stalled polymerase would arrest and would start incorporating and removing nucleotides opposite the lesion. This would

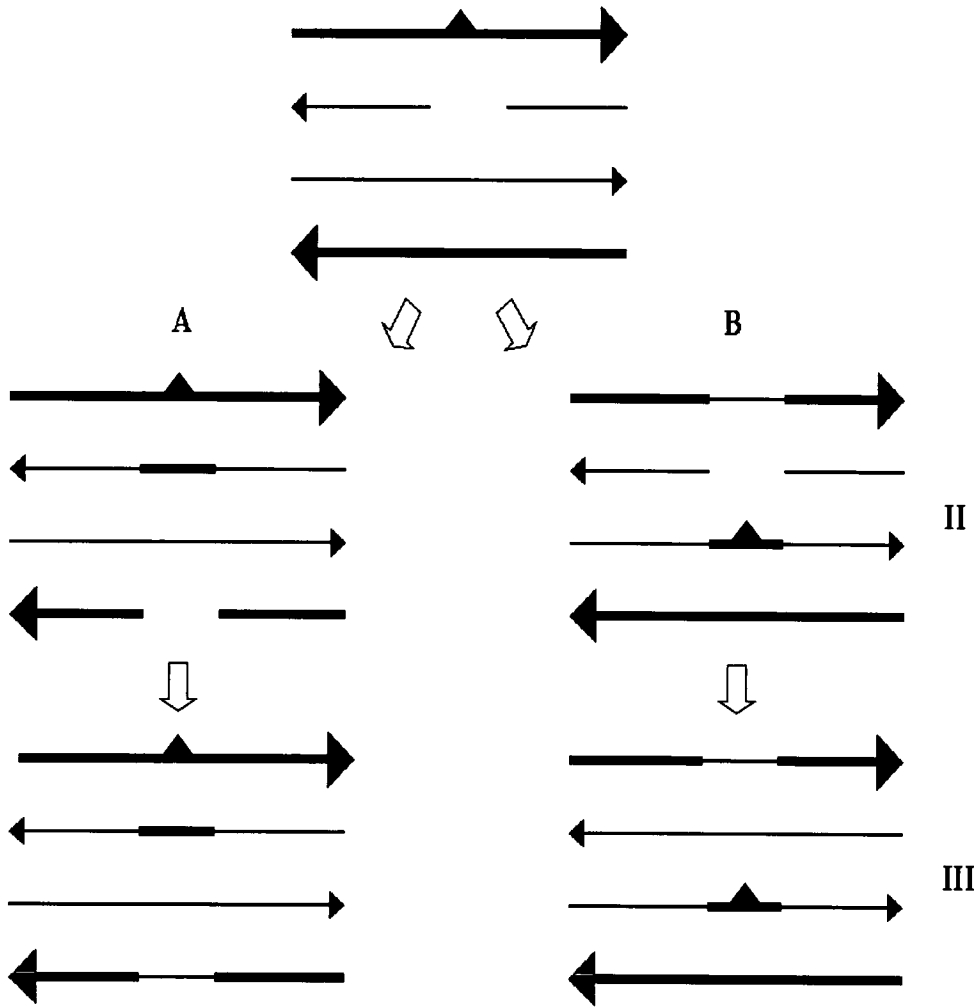


Figure 1.6: Schematic view of the postreplication repair mechanism in bacteria. Thick lines represent parental DNA containing dimers (solid triangles). Thin lines represent daughter DNA synthesised after irradiation. Two different exchange processes occur with equal probability: (A) The gap is filled in with isopolar undamaged parental DNA, which creates a gap in this latter DNA (IIA). This gap is later filled by repair synthesis (IIIA). Ultimately, the dimer remains in the parental strand. (B) The region containing the dimer is exchanged with the isopolar daughter strand, creating a dimer-free template opposite the gap (IIB) which can be used to direct the filling of the gap (IIIB). Ultimately, the exchanged dimer remains in the daughter strand. Adapted from Meneghini *et al.*, 1981.

in turn induce a SOS mechanism which somehow suppresses the exonuclease activity of the polymerase, thus resulting in replication past the lesion with an increase in the possibility of error (for a detailed description of the SOS response see Friedberg *et al.*, 1995, Chapter 10). In *E. coli*, translesion synthesis is thought to be carried out by Pol

III together with at least three accessory proteins, the SOS-controlled umuDC gene products and the activated form of RecA (Friedberg et al., 1995; Woodgate and Levine, 1996). However, translesion synthesis in *E. coli* also has an umuDC-independent component, which points towards an underlying higher complexity of this strategy (Napolitano et al., 1997; Baynton et al., 1998). Apparently, there is an indication that, in bacterial systems, recombination repair predominates over translesion synthesis (Ganesan and Seawell, 1975; Woodgate and Levine, 1996).

In budding yeast, *S. cerevisiae*, the components of the RAD6 pathway seem to be responsible for most of the yeast's resistance to DNA damage and for almost all induced mutagenesis (Baynton et al., 1998). Although less than half the genes involved in the replication of damaged DNA have been cloned, recent studies have allowed the identification of protein functions that participate in this pathway. The heterodimeric complex Rad6-Rad18 has been proposed to recognise single-stranded DNA associated with stalled replication forks at damaged sites (Baynton et al., 1998). Once recruited, the complex may ubiquitinate components of the replication machinery, promoting replication recovery. Translesion replication in *S. cerevisiae* is thought to be carried out by DNA polymerase ζ , which contains one catalytic subunit encoded by the gene REV3 and at least one other gene product encoded by the REV7 gene and has been shown to be able to replicate through damage sites (thymine-thymine dimers) (Nelson et al., 1996). The REV1 gene product, which has a weak homology with the *E. coli* umuC protein (Nelson et al., 1996), has been shown to insert cytosine preferentially across from a template abasic site, creating a lesion terminus that can be easily extended by Pol ζ (Baynton et al., 1998). In addition, Rev3 DNA polymerase has recently been shown to be required for translesion synthesis *in vivo* (Baynton et al., 1998).

1.4.2 Replication on a damaged template in mammalian cells

It is apparent that despite the existence of lesions in the replicating DNA, the DNA synthesis machinery still manages to proceed with replication. In fact, as discussed before, 37% of XP cells containing 16000 unexcised dimers per genome are able to survive and replicate. Direct proof that replication can proceed in the presence of DNA lesions, comes from experiments performed with CHO cells. When UV-irradiated, replicated

and unreplicated DNA derived from these CHO cells contain the same amount of dimers, so the replication machinery must have bypassed the lesions.

In this same experiment it was possible to determine that these cells carry out a minimal amount of dimer excision (about 30% in 24 hours after a dose of 5-10 J/m²) and that this process does not preferentially take place ahead of the replication fork as a way of clearing the way for the replication machinery (Meyn et al., 1974). The same is true for normal human fibroblasts although these cells are better at removing the dimers (approximately 50% in 24 hours) than their rodent counterparts (Waters, 1979). An account of the different mechanisms used by mammalian cells to bypass the lesion-induced replication arrest and the factors that influence this recovery is given next.

1.4.3 Factors that influence recovery from DNA replication arrest after DNA damage

Studies have shown that DNA replication in mammalian cells is inhibited by UV light (Cleaver, 1965; Klimek and Vlasinova, 1966). In rodent cells the extent and duration of DNA synthesis blockage is both cell-line and fluence-dependent (Griffiths and Ling, 1989) and the same is the case with human cells. Wild type human cells do not show significant blockage following exposure to 2.5 J/m², but after exposure to 5 J/m², they show significant blockage as shown by DNA fibre autoradiography (Griffiths and Ling, 1989). This arrest lasts for 5 hours after which the segment lengths return to control values. Excision repair-deficient human cells, on the other hand exhibit significant blockage both immediately and 5 hours after irradiation with the low UV dose (2.5 J/m²).

DNA excision repair has been proposed as the main agent behind the removal of the dimers (Klimek and Vlasinova, 1966; Cleaver, 1965) and the recovery from DNA synthesis arrest. However, fibroblasts derived from patients with Cockayne's syndrome, show no recovery of DNA synthesis after UV, even though these cells can carry out excision repair of dimers (Lehmann et al., 1979). This suggests that although DNA excision repair is necessary for the recovery of DNA synthesis after UV irradiation it is not sufficient. Are there any other factors that contribute to that recovery?

The rate of recovery of DNA synthesis after UV depends on several factors:

1. the number of cells in S phase,
2. the number of active replicons per cell
3. the size of the replicons (Cleaver et al., 1983)
4. the rate of DNA elongation and
5. the excision repair capacity of the cells (Cleaver et al., 1983).

Cells tend to accumulate in S phase after UV irradiation, but their number only decreases after recovery has taken place, so DNA recovery must take place in cells that are in S (going through active DNA replication). Also, if the number of active replicons would increase after UV it could lead to a recovery in the rate of DNA synthesis. Finally, a recovery in the rate of DNA replication would also be achieved if cells acquired the ability to replicate lesion-containing templates more efficiently.

Cycloheximide has been reported to induce a significant reduction in the rate of DNA synthesis recovery. However, a specific inhibitor for DNA synthesis, fluorodeoxyuridine, has no effect on recovery after UV (Lehmann, 1979). This suggests that protein synthesis is necessary to achieve DNA synthesis recovery and that this is done through a pathway different from excision repair, since excision repair does not require protein synthesis.

This situation is different in CHO cells. These cells seem to require DNA synthesis to reach the recovery state. When CHO cells are synchronised in G1 and irradiated with 5 J/m^2 of UV, their rate of DNA synthesis (as measured by the rate at which DNA achieves hybrid buoyant density, in the presence of BrdU) drops to 70% of the control in the first S phase but becomes normal in the second S phase. Holding the cells in G1 does not increase the rate of recovery, so in order to achieve DNA recovery, it seems necessary that the cells go through an S phase. Only 25% of the dimers are eliminated in these cells after 5 J/m^2 , so excision repair does not seem account for such recovery (Meyn et al., 1977).

It is possible that recovery is in fact due to both excision repair and/or an inducible mechanism(s) that may involve *de novo* protein synthesis. Normal human fibroblasts have both mechanisms and they recover efficiently and fast. In the case of rodent cells, their repair capacity is very poor and they would be more dependent on the inducible process(es) (Meyn et al., 1977; Meneghini et al., 1981). But, what happens when NER is not functional? What other mechanisms can account for the recovery of DNA replication after the DNA damage-induced arrest of DNA synthesis in rodent cells? A review of these alternative processes is given below.

1.4.4 Dealing with unexcised lesions: Mechanisms of replication arrest recovery

In general, the process by which cells manage to replicate DNA that contains lesions is known as postreplication repair (PRR). PRR is used here as an equivalent term for elongation of DNA synthesis on damaged templates. PRR has been conventionally seen by alkaline sucrose gradients. Cells are grown in the presence of radioactive thymidine for a short time and then put in normal medium for different amounts of time. The centrifugation of the DNA of these cells in the alkaline sucrose gradients reveals an increase in the size of the nascent DNA strand with time. In UV-irradiated cells, this elongation is delayed as compared to the unirradiated controls, and this delay has been shown to be most likely due to the presence of lesions on the parental DNA strands. PRR has been shown to occur in a variety of cells and by techniques other than sucrose sedimentation gradients (Meneghini et al., 1981).

Cordeiro-Stone *et al.* showed that SV40-transformed XP cells that had been irradiated had a lower rate of DNA elongation than the control cells (Cordeiro-Stone et al., 1979). Similar results have been obtained using DNA fibre autoradiography to study the inhibition and recovery of DNA synthesis in V-79 Chinese hamster cells (Dahle et al., 1980): a reduced rate of fork displacement was detected in irradiated V-79 cells, suggesting that dimers do constitute a temporary block for the DNA replication machinery during the first 5 hours after exposure. Within 10 hours, however, the lesions are apparently repaired or modified (or the replicative enzymes altered) so that DNA chain growth is no longer arrested (Dahle et al., 1980).

Different cells may have different types of PRR. One case is that of cells derived from patients suffering from an XP variant form. These patients are clinically identical to XP patients but their cells show normal capacity for NER of dimers. Apparently, these cells have an impaired PRR, and are most likely defective in the process of translesion synthesis (Cordonnier et al., 1999).

DNA synthesis inhibitors such as hydroxyurea, cytosine arabinoside, and excess of thymidine are also inhibitors of PRR. This indicates that PRR and DNA synthesis may share some enzymatic components. Caffeine and theophylline are very specific inhibitors of PRR. They do not have an effect at moderate doses in untreated control cells, but they specifically inhibit DNA elongation at moderate doses in rodent cells treated with DNA-damaging agents. In human cells inhibition of PRR by caffeine is not observed, with the exception of XP variant (Lehmann et al., 1975; Meneghini et al., 1981).

The PRR mechanism(s) would involve a recombination process between the damaged region of the template and an homologous homopolar sequence of DNA which has not been damaged, therefore avoiding the direct replication of the damaged DNA. This process or processes (referred to as mechanism(s) of alternative copy) would not be mutagenic. Another way of dealing with unexcised lesions would be the direct replication of the portion of DNA containing the lesion (referred to as translesion synthesis). This type of repair would be expected to be highly mutagenic.

Mechanisms of alternative copy

Recombination

Techniques successfully employed in bacterial cells to demonstrate the existence of recombinational processes failed in mammalian cells (Lehmann, 1972; Meneghini and Menck, 1978).

A more direct approach to the problem was taken by Meneghini *et al.* who showed that in fact there was evidence for a very low-frequency recombinational process between the daughter and the parental strands of DNA (refer to Fig. 1.6) whose frequency is reduced to even lower levels as time after irradiation increases (Meneghini et al., 1981).

Meneghini proposes that this mechanism is probably active soon after the damage is inflicted and another mechanism takes over at later times. Proof for the existence of recombinational processes also comes from the strand break rejoining process in mammalian cells, which has biphasic kinetics. It has been suggested that the fast component of this process reflects the direct DNA strand rejoining, whereas the slow component reflects in fact recombinational processing (Friedberg et al., 1995, Chapter 12).

Branch migration

This mechanism also involves recombination with an alternative isopolar template (see Fig. 1.7). If the replication fork reaches a lesion the daughter strand at the opposite lesion-free template continues replication for a certain period of time, leaving behind the other daughter strand halted at the lesion and a single strand region ahead of it. Branch migration then takes place, bringing back the replication fork to the point of the lesion and allowing the blocked daughter strand to switch templates from parental strand to the isopolar daughter strand which had already gone ahead and undergone replication.

However, direct attempts to demonstrate the presence of gaps in the daughter strand opposite CPDs to provide evidence for the PRR model have been unsuccessful (Meneghini, 1976; Clarkson and Hewitt, 1976). There are therefore, doubts as to the precise position of those gaps in relation to the lesion and whether the gaps are formed only in the lagging strand (Meneghini et al., 1981), or in both the leading and the lagging strand (Lehmann, 1979).

Translesion synthesis

In this process a direct copy of the damaged template takes place. As opposed to recombination and branch migration this mechanism is supposed to be highly mutagenic. If translesion synthesis takes place one would expect to find cells that survive UV irradiation with a high number of mutations per cell. Studies carried out in the HGPRT locus of excision-defective XP cells point towards this possibility (Meneghini et al., 1981). Mammalian fibroblasts in culture have a high probability of surviving

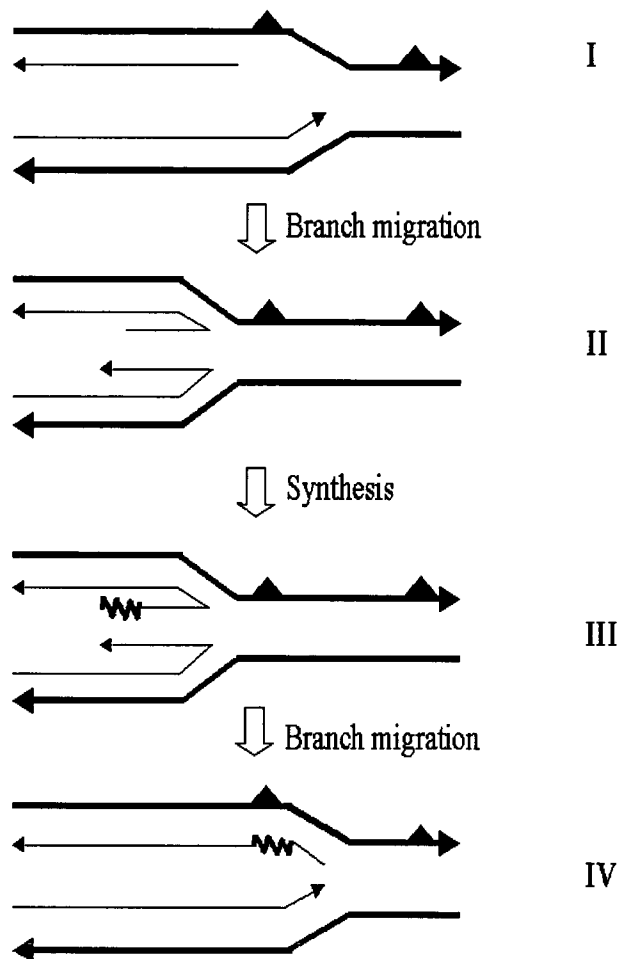


Figure 1.7: Schematic representation of the branch migration mechanism. When the fork reaches a lesion (represented by the solid triangles) in the parental strand, replication of the daughter strand terminates while replication of its sister continues for a short distance (I). Then by branch migration, the parental strands reanneal, displacing the daughter strands which in turn hybridise with each other (II). The longer sister strand then serves as a template for the shorter one. After synthesis occurs, branch migration proceeds in the reverse direction until the normal replication fork is formed and fork movement can proceed (III, IV). Adapted from Doniger, 1978.

when their genome contains up to 50000 unexcised dimers, which means that they can actually tolerate such a burden of mutations (Meneghini et al., 1981). In addition, excision-repair deficient *E. coli* produces point mutations after a dose of 0.6 J/m^2 with a frequency that is an order of magnitude lower than that of mammalian cells (10^{-4} in bacteria compared to 10^{-3} in mammalian cells). This seems to point to the fact that

translesion synthesis is a common feature in mammalian cells. The human and mouse homologues of the yeast *S. cerevisiae* REV3 gene, which encodes the catalytic subunit of DNA polymerase ζ , have been recently cloned (Gibbs et al., 1998; van Sloun et al., 1999). The human REV3-homolog, hREV3, maps to the chromosome 1p32-33 and encodes an mRNA of approximately 10 Kb, with variable expression depending on the tissue examined (Xiao et al., 1998). The human gene encodes an expected protein of 3,130 residues (Lin et al., 1999), about twice the size of the yeast protein (1,504 aa). The sequence of the expected protein suggests that it is the catalytic subunit of a DNA polymerase of the pol ζ type (Gibbs et al., 1998; Lin et al., 1999).

Joining of adjacent replicons

A lesion may function as a premature termination point for a replicon (Park and Cleaver, 1979). This fork could be relieved by chain growth from the adjacent unblocked fork. This model would provide an explanation for the observed long term interruptions in the daughter strands, which would occur when two forks approaching from adjacent origins are blocked at two distinct dimers. This model also has the advantage of not requiring the formation of gaps for it to be plausible. However, the evidence points towards the existence of such gaps. Meneghini proposes a modification of Park and Cleaver's model in which the confluence of adjacent forks, one being blocked by a dimer, causes the formation of gaps (see Fig. 1.8). This theory has later been put forward by other authors (Spivak and Hanawalt, 1992; Painter, 1985)

1.4.5 Co-regulation of DNA synthesis and DNA repair

It is not known how DNA damage inhibits DNA replication and yet allows DNA repair. Proteins such as replication protein A (RPA, also known as human single-stranded DNA-binding protein, HSSB), proliferating cell nuclear antigen (PCNA), activator 1 (A1, also known as replication factor C, RFC) and DNA polymerase δ (pol- δ) have all been implicated in both processes.

When normal cells are exposed to genotoxic agents, the levels of p53 tumour-suppressor protein rise, inducing p21 Cip1/Waf1 (p21) (El-Deiry et al., 1993). p21 has two functions. First it is an inhibitor of cyclin-dependent kinases, possibly causing cells to

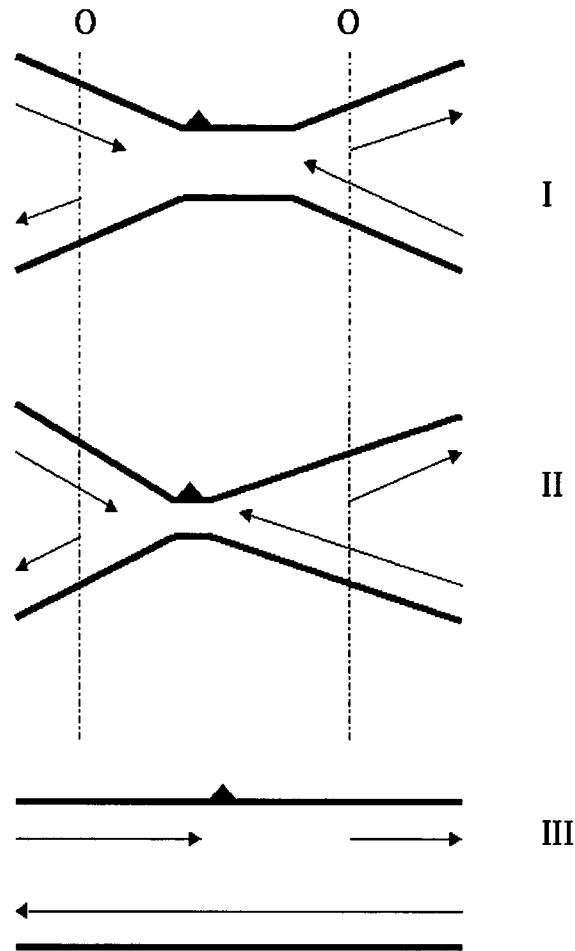


Figure 1.8: Schematic representation of the joining of adjacent replicons. Replication is assumed to be discontinuous at the lagging strand and O represents the Origin of Okazaki fragments. The replication fork at left is blocked at the dimer (represented by a solid triangle), whereas the one at the right is moving toward the dimer (I). After reaching the block (II) DNA synthesis resumes in the lesion-free template but remains arrested on the lesion-containing template, giving rise to the gap (III).

arrest at the G1 phase of the cell cycle (Cox, 1997; Lin et al., 1996)(see next sections: Cell Cycle Control I, section 1.5 and II, section 1.6). Secondly, it interacts with PCNA to inhibit DNA replication without affecting PCNA function (Cayrol and Ducommun, 1997; Chen et al., 1996b; Jackson et al., 1995; Li et al., 1994b; Waga et al., 1994).

There is evidence to suggest that cells exposed to UV irradiation are inhibited in the synthesis phase (S), rather than at the G1-S boundary (see next sections: Cell Cycle

Control I , section 1.5, and II, section 1.6). DNA synthesis in UV-irradiated cells is significantly inhibited while repair occurs (Lu and Lane, 1993) and HeLa cell extracts show reduced capacity to support DNA replication *in vitro* as the level of UV-induced S-phase arrest increases (Carty et al., 1994).

In fact, S-phase arrest occurs not only by a decrease in replicon initiation but also by inhibition of the replication-fork progression (Lehmann et al., 1975; Lehmann, 1981; Park and Cleaver, 1979; Kaufmann et al., 1980; Painter, 1985). Inhibition of replicon initiation after UV is actively regulated by a trans-acting mechanism, but it is not clear what the mechanism behind inhibition of chain elongation is, nor whether a cis effect caused by the damage in the replication fork itself plays a role in replication arrest (Ryan et al., 1994). The fact that S-phase human cells arrest for long periods of time after UV before proceeding with the cell cycle suggests that the presence of damage in S-phase cells is recognised and that the inhibition of DNA replication is actively controlled (Wang and Ellem, 1994).

The arrest of replication-fork progression after UV irradiation has been shown to occur *in vivo* in chromosomal DNA by DNA-fibre autoradiography (Edenberg, 1976; Griffiths and Ling, 1989) and in SV40 minichromosomes by electron microscopy (Berger and Edenberg, 1986). However, the duration of the fork arrest, the mechanism of the recovery from the arrest and the detailed mechanism by which UV inhibits replication-fork movement are not known. Just as an example, estimates for the duration of the fork arrest, range from 15 minutes (van Zeeland and Filon, 1982) to several hours (Griffiths and Ling, 1989; Griffiths et al., 1990; Lehmann et al., 1975; Park and Cleaver, 1979). An account of the most recent advances in the understanding of the regulatory mechanisms behind the control of the cell cycle progression both through M and S, is presented in the Cell Cycle Control sections.

1.5 Cell Cycle Control I: Progression through M

Each time a cell needs to divide, the cellular machinery has to make a decision on whether to carry on with mitosis or not, and this decision is indeed under very tightly regulated mechanisms (Roberts et al., 1994). In order to decide, the cell has to take

into consideration a wide variety of signals that indicate when division is needed and appropriate. It is now known that these signals come from two different sources: signals that come from the extracellular environment (Graña and Reddy, 1995), and signals that come from intracellular checkpoint controls. Environmental signals provide the cell with information on the available nutrients, whether there is a need to divide, and whether there are overriding reasons not to begin a replicative cycle. Signals from internal checkpoints make sure that the preceding steps of the cell cycle have been successfully completed before the next stage of the cell cycle takes place (Murray, 1994). Five cell cycle control checkpoints have been identified to date (Lees and Harlow, 1995):

1. A checkpoint that monitors for DNA damage and arrests cells in G2
2. A checkpoint that monitors for DNA damage and arrests cells in G1
3. A checkpoint that signals that DNA synthesis has been completed before mitosis begins
4. A checkpoint that monitors completion of mitosis before allowing entry into S phase
5. A checkpoint that ensures that the chromosomes are correctly aligned at the metaphase plate prior to the initiation of the anaphase.

Most of the regulatory mechanisms of cell cycle control act during the gap or G phases of the division cycle. Thus, the G1 phase between mitosis (M phase) and DNA synthesis (S phase) and the G2 phase between DNA synthesis and mitosis are the times in which the regulatory decisions of the cell cycle are made. In this section I will focus primarily on the regulation of M phase and the mechanisms underlying the G2/M transition as well as the regulatory connection between the S phase and the M phases of the cell cycle. In the next section I will deal with the regulation of the S phase in more detail.

The current model of mitotic regulation in vertebrates is shown in Fig. 1.9 (Basi and Draetta, 1995). Briefly, during interphase, cdk1 (cyclin-dependent kinase 1) and cyclin B associate and are phosphorylated on Thr14, Tyr15 and Thr161. This inactive complex (preMPF) accumulates during S and G2. This complex becomes active by

the dephosphorylation of cdk1 on Thr14 and Tyr15, which is the rate-limiting step controlling entry into mitosis. Once this happens mitosis starts. Inactivation of MPF is brought about by the ubiquitination and subsequent degradation of cyclin B. Inactivation of MPF is necessary for the exit from mitosis. After degradation, dephosphorylation of Tyr161 takes place, which renders a monomeric, inactive (unphosphorylated) cdk1 and the cycle starts again (Basi and Draetta, 1995; Coleman and Dunphy, 1994).

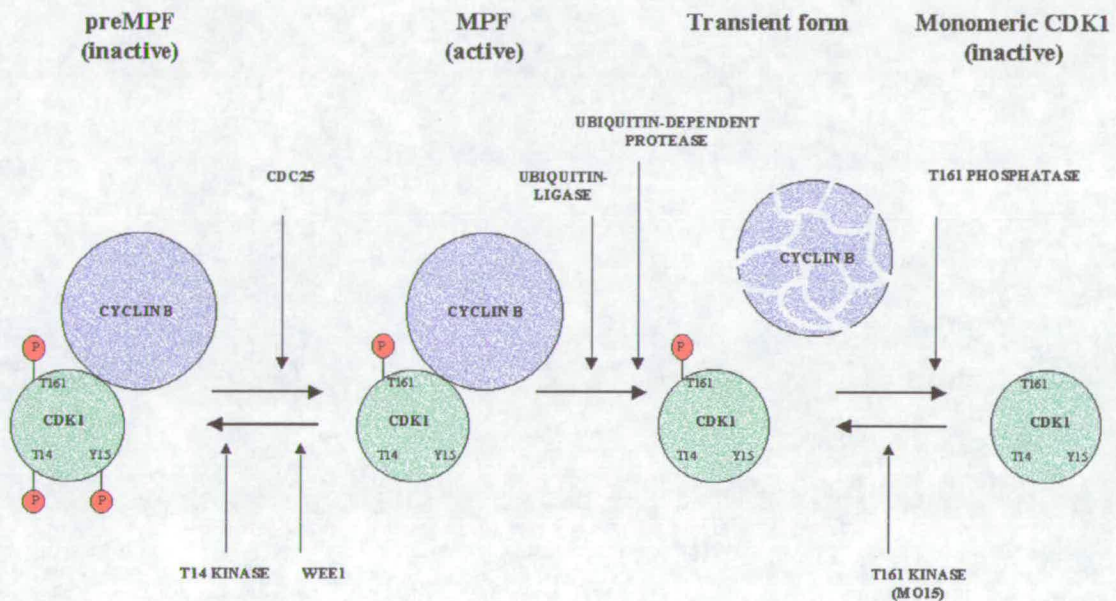


Figure 1.9: Activation and deactivation of cdk1 at mitosis. Dephosphorylation of cdk1 on Thr14 and Tyr15 promotes activation of the cdk1–cyclin B complex formed during interphase (preMPF) and is the rate-limiting step for mitotic initiation. The Thr161 phosphate regulates positively the cdk1 kinase and is also present in the active M-phase promoting factor (MPF). At the metaphase–anaphase transition, cyclin B is ubiquitinated and subsequently degraded by an ubiquitin–dependent protease. Following degradation of cyclin B, cdk1 is dephosphorylated on Thr161 and MPF is inactivated. After formation of inactive monomeric cdk1, cells can exit from mitosis and start a new cycle. Adapted from Basi and Draetta, 1995.

PCNA and p21

The interaction between cell cycle regulators and other molecules that take part in repair processes, such as p21 and PCNA, is of special interest in the context of this

thesis. Several cdk–cyclin complexes are found to associate with PCNA and with small inhibitory peptides, which range in size from 14 to 27 kDa. In mammalian cells, the cdk1–cyclin B complex associates with PCNA and a 21 kDa protein (p21). Interestingly, this association is absent in transformed cells. As seen in the DNA Replication section 1.2, PCNA is a cofactor of DNA polymerase δ , and it is required during DNA replication and repair (see Replication section 1.2 and Repair section 1.1 for further information on PCNA). p21 gene was cloned simultaneously by 4 different groups (Graña and Reddy, 1995; Elledge and Harper, 1994), which named the protein in four different ways according to the approach they used to clone it. The first group was working on the isolation of proteins that interacted with cdk2 and named the gene CIP1 (cdk2–interacting protein-1). The second group were screening for genes induced by p53 and named it WAF1 (wild type p53–activated fragment-1). A third group, which was cloning genes specifically activated during senescence, called this gene SDI1 (senescent cell–derived inhibitor-1). The fourth group microsequenced a 21 kDa protein that co–immunoprecipitated with cyclin D. p21 was capable of inhibiting the catalytic activity of all the cdk–cyclin complexes tested *in vitro*. It is interesting to note that, *in vitro*, the cdk1–cyclin B complex is inhibited by p21 less efficiently than the cdk2–cyclin A complex (Basi and Draetta, 1995). p21 also inhibits the cdk2–cyclin E complexes necessary for the early events in DNA replication (Jackson et al., 1995; Hartwell and Kastan, 1994). Structural studies of the p21 protein have provided an explanation based on conformational changes for the mechanism that regulate the binding diversity of p21 (Kriwacki et al., 1996).

The presence of PCNA in the cdk1–cyclin complex is not well understood. It has been proposed that the function of PCNA may be to signal the presence of DNA damage or other signals. The fact that this complex of PCNA, p21 and cdk1–cyclin B does not exist in transformed cells where S and M are still coupled, may indicate that it plays an important role in the process of cell transformation, but that it is probably not essential for the regulation of MPF (Basi and Draetta, 1995).

1.5.1 The dependence of mitosis on S phase

The succession of the different cell cycle phases is regulated in eukaryotes by the cell cycle checkpoints. Checkpoints are molecular controls which act at specific points of the cell cycle and prevent entry into the subsequent phases of the cell cycle if the preceding phase has not been properly completed. Thus, in order to proceed into mitosis, a checkpoint control monitors that S phase has been completed. If DNA damage is present, for example, entry into mitosis is delayed until the DNA has been repaired or DNA replication has been completed. However, little is known about the precise mechanisms which monitor the presence of unreplicated or damaged DNA, and how this signal is then transmitted to the cell cycle regulation machinery (Basi and Draetta, 1995).

In a normal cell cycle, a type-2A phosphatase, regulating the cdc25 and the weel activities, is gradually inactivated during the G2 phase (Basi and Draetta, 1995). Due to the balance between the activity of kinases and phosphatases, the inhibition of the type-2A phosphatase results in the inactivation of weel and the activation of cdc25. cdc25 then induces the dephosphorylation of cdk1 (Coleman and Dunphy, 1994), which brings about the activation of preMPF and entry into mitosis. If DNA synthesis is inhibited, a feedback control mechanism blocks inactivation of the type-2A phosphatase until DNA replication is completed (Basi and Draetta, 1995). Another checkpoint could be in charge of controlling the activity of weel by regulating the kinase which phosphorylates and inactivates weel (Coleman and Dunphy, 1994). Furthermore, it has been suggested that it is possible that other checkpoints exist which are independent of the regulation of cdk1 on tyrosine. In this model, PCNA and p21 could function as signalling molecules detecting DNA damage (Basi and Draetta, 1995).

G2/M DNA damage checkpoint

The G2/M checkpoint monitors completion of DNA synthesis and the presence of DNA damage. This is a replication-dependent checkpoint which prevents mitosis by inactivating cdk1 (Lees and Harlow, 1995). Delay in passage through G2 results in a postponement of mitosis and cell division, and has been observed in all eukaryotic organisms examined, including ciliates, fungi, algae, flowering plants, slime moulds,



sea urchins, insects and mammals (Rowley, 1998).

Early studies of G2 delay in mammalian cells resulted in the accumulation of a number of data that can be summarised as follows (Rowley, 1998):

- Irradiation of growing cells results in a delay in entering mitosis, which depends on the radiation dose (55 min delay/Gy in Chinese hamster ovary cells), the degree of cellular differentiation (mouse intestinal crypt cells) and the phase of the cell cycle (V79 hamster and HeLa cells).
- From experiments with HeLa cells it was seen that once the cell has entered mitosis, it is no longer delayed in that cell cycle.
- Studies carried out with CHO cells to pinpoint the precise position of the G2 arrest in the cell cycle have shown that G2 delay happens 49 minutes before completion of cell division, 17 minutes before metaphase and 18 minutes after cycloheximide transition point (the point in the cell cycle after which this protein synthesis inhibitor cannot delay entry into mitosis).

The first attempts to characterise the molecular mechanisms behind the G2 arrest showed that G2 delay required protein and mRNA synthesis (Rowley, 1998). Studies performed using caffeine (which was known to sensitise bacteria to UV-induced damage) showed that mammalian cells were also sensitised to radiation upon treatment with caffeine, and CHO cells exposed to it immediately after irradiation are not delayed in G2 (Rowley, 1998). What is more, if caffeine is administered to CHO cells immediately after irradiation and then removed after an interval of as long as 12 hours, a period of delay follows that is identical in length to that observed in cells that have not been treated with caffeine. It seems that caffeine not only blocks the expression of the delay, but also preserves the damage that gives rise to it, allowing the expression of delay on caffeine removal. Caffeine may be sensitising these cells to DNA damage at two levels: indirect inhibition of repair via abolition of checkpoint controls and direct inhibition of repair functions, perhaps by binding sites of damage, as hypothesised for UV-irradiated bacteria (Rowley, 1998).

No mammalian cell mutant has yet been identified that is defective for G2 delay alone,

so additional drug treatments and other manipulations are required for the determination of the effects of losing the G2 checkpoint. When caffeine treatment is combined with the method of mitotic selection (mammalian cells usually round up upon entry into mitosis, so it is usually possible to select for mitotic cell subpopulations by shaking the culture flask and selectively detaching cells in M) it is possible to measure survival after irradiation of those cells that were treated in G2 and delayed or failed to delay at G2. Cells that received caffeine immediately and continuously after irradiation (X-rays) were radiosensitive to killing and showed the same radiosensitivity as mitotic cells. These cells also showed increased number of achromatic gaps, deletions and exchanges. However, if an interval was allowed before the addition of caffeine, permitting a period of delay, aberration frequencies declined with the length of the interval (Rowley, 1998). Assuming that chromosome aberrations result from DNA double-strand breaks, and because caffeine prevents the repair of chromosomal aberrations, the caffeine should reduce the rate of DSB repair. This seems to suggest that the G2 checkpoint, DNA damage repair and chromosomal integrity are intimately related. Indeed, it has been shown that DNA damage itself, i.e., the induction of a single DSB in the genome of the budding yeast, is sufficient to induce a cell cycle delay, arguing for the fact that DNA damage does initiate the G2 arrest and that the delay provides time for the repair of DNA damage, prior to chromosome segregation (Rowley, 1998). Consistent with this result, Chinese hamster cells deficient in the repair of DSBs suffer abnormally long delays in G2 (Rowley, 1998).

The proteins involved in the G2 delay biochemical pathway are likely to include *cdc25*, *cdk1* itself, or the tyrosine kinase *wee1*. PCNA and p21 may also exert an additional regulatory role on *cdk1*, but this still remains a hypothesis and the pathway that PCNA/p21 use to regulate *cdk1* also remains uncharacterised. The fact that these two factors play a key role in the S phase regulation, makes their possible involvement in the coupling of S and M a plausible option (see Replication, section 1.2 and Cell Cycle Control II, section 1.6). Studies of specific overexpression of p21 in mouse liver, where hepatocytes overexpressing p21 showed a phenotype that included increased levels of polyploidisation, led Wu *et al.* to postulate that the p21-overexpressing hepatocytes may be arrested at G2 and that p21 may be playing a role in the mechanism of this delay (Wu *et al.*, 1996). The fact that the phenotype of the liver of the p21-overexpressing

mice closely resembles that of the ERCC1-deficient mice may also provide an additional link between p21, DNA repair (NER) and control of the cell cycle at G2.

1.6 Cell Cycle Control II: Progression through S

1.6.1 Initiation events within S phase

It is possible that replication might be controlled in the cell cycle by limiting the availability of the factors required at the replication fork. However, most replication factors are present in the cell throughout the cell cycle. This is due in part to the fact that many of the factors involved in replication are also involved in other processes, such as DNA repair. This probably means that replication must be controlled by a relatively small number of S phase-inducers whose function is to regulate when and where initiation of replication takes place⁸.

Through fusion studies it was possible to determine that S phase-inducers can only act on G1 nuclei and cannot induce G2 nuclei to undergo another round of DNA replication. In S phase/G2 and G1/G2 HeLa cell fusions the G2 nuclei did not replicate until after passage through mitosis, which suggested that the absence of DNA replication in normal G2 cells is not due to the presence of diffusive inhibitors in the G2 cytoplasm and that there were specific mechanisms for the prevention of re-replication of the G2 nuclei (Blow, 1995)(see below).

G1 and S phase nuclei in G2 cytoplasm replicated normally. This suggests that progression of events in S phase does not require cytoplasmic factors to proceed in an ordered fashion. What is more, fusion of G1 and late S cells caused the G1 nuclei to enter S phase prematurely, with replication patterns of nuclei in early S phase, rather than in late S phase (Blow, 1995). This suggested that the S phase-inducers present in the cytoplasm induce responsive nuclei to undergo the normal sequence of S phase event, but are not themselves responsible for dictating the stage of replication the nucleus should undergo.

⁸ For a complete review on the subject of cell cycle control, it is recommended to read *Cell Cycle Control*, Hutchinson and Glover editors, 1995. The data for this section have been taken from Blow, 1995.

In summary, replication control can be divided in two distinct components: the DNA template in the nucleus (see Replication section 1.2), and activities in the cytoplasm which act on this nuclear template. The nucleus can be in two different states: responsive or not responsive to the S phase-inducers by undergoing DNA replication. Also, the cytoplasm provides two types of activity, one that induces a responsive (G1) nucleus to undergo S phase (S phase-inducers), and another (related to passage through mitosis) that changes a refractory G2 nucleus into a responsive G1 nucleus (Blow, 1995). Once replication is initiated, the S phase proceeds through the different stages of DNA replication (including initiation at early- and late-firing origins) without the need for specific signals from the cytoplasm.

1.6.2 Prevention of re-replication in a single S phase

As mentioned before, fusion experiments on HeLa cells showed that only G1 nuclei can initiate DNA replication in response to cytoplasmic signals. G2 nuclei on the other hand must go through mitosis in order to regain responsiveness to these signals. However, if G2 nuclei, in a *Xenopus* cell-free system, were allowed to progress into mitosis and undergo subsequent nuclear envelope breakdown and chromosome condensation, they were then capable of undergoing a further round of replication. Some metaphase function therefore permitted G2 nuclei to revert to the responsive G1 state (Blow, 1995). What is more, agents that caused nuclear envelope permeabilisation, such as lysolecithin or phospholipase, rendered the G2 nuclei responsive to the S phase-inducers and capable of re-replication in fresh extract (Blow, 1995).

A model has been put forward that explains these results (Blow, 1995; Blow, 1993). An essential replication factor called 'Licensing Factor', can bind to the DNA during late mitosis or early interphase before nuclear assembly has occurred. Since Licensing Factor cannot cross the nuclear envelope, once the nuclear assembly is complete it is only present in the nucleus where bound to DNA. On entry into S phase, each molecule of Licensing Factor supports a replication initiation event after which it is destroyed or inactivated. Therefore, in G2 there is no active Licensing Factor present in the nucleus, and the nuclear envelope has to be transiently permeabilised to allow a further round of DNA to happen.

Experiments using *Xenopus* extracts have also shown that Licensing Factor activity can be inhibited by the addition of the protein kinase inhibitor 6-dimethylaminopurine (6-DMAP) prior to exit from metaphase, resulting in a block of the initiation of replication (Blow, 1995; Blow, 1993). Licensing Factor levels are low during the early stages of mitosis and they rise suddenly shortly after the metaphase–anaphase transition, allowing the decondensing telophase chromosomes to become licensed before completion of nuclear assembly. This abrupt appearance of Licensing Factor does not require protein synthesis, so it has been proposed that Licensing Factor is in fact a post-translational modification of a protein that is already present (Blow, 1995). This post-translationally modified protein is probably the real target of 6-DMAP (Blow, 1993). Licensing Factor that remains cytoplasmic and has not bound DNA probably becomes unstable and is degraded by mid-S phase, where it has disappeared.

However, there is little evidence on the molecular mechanisms of DNA re-replication control. Studies with drugs that induce chromosome replication in the absence of intervening mitosis, such as trichostatin A, leptomycin B and staurosporine-analogue protein kinase inhibitors, have provided one line of experimental evidence on this mechanism in mammalian cells. Uncoupled cell cycle without mitosis has been reported to occur through the action of a chemical that inhibits protein kinase activity, K-252a (Usui et al., 1991). Treatment of rat diploid fibroblasts with K-252a results in the accumulation of cells with high ploidy values (8C and higher, depending on the cell line used), and concomitant with this, although weak cdk1 activity is seen during S, cdk1 activity is not detected during M. Thus, it appears that cdk1–cyclin B and cdk1–cyclin A complexes may play a role in the control of the re-replication process (Kearsey et al., 1996), and indeed, there have suggestions that cdk1 may be the responsible factor for what has been termed ‘G2 memory’ (Usui et al., 1991). Briefly, G2 memory is what tells cells that replication (S phase) has taken place and that, in the absence of mitosis, should not happen again. These observations are supported by genetic experiments in fission yeast which have shown that cdk1–cyclin B complexes mark the cell in the G2 state (O’Connell and Nurse, 1994). Temperature sensitive mutations in *cdc13* (a B-type cyclin) or *cdk1* cause increase in ploidy resulting from multiple S phases without mitosis (O’Connell and Nurse, 1994). Deletions of the *cdc13* gene cause the cells to accumulate with grossly enlarged nuclei containing up to a 32C DNA content (Hayles

et al., 1994).

Cyclin G and p21 have also been implicated in the regulation of G₂/M transition in primary cultured and immortalised rat fibroblasts in response to DNA damage (Shimizu et al., 1998). What is more, there is increasing evidence that p21, along with the mitotic kinases cdk2 and cdk1–cyclin B complexes, plays a role in the control of re-replication (Bates et al., 1998; Guadagno and Newport, 1996). Absence of p21 has been shown to cause diploid cell lines derived from the human colorectal cancer cell line HCT116 to undergo re-replication in the presence of DNA damage (Waldman et al., 1996). These cells acquire grossly deformed, polyploid nuclei and subsequently die through apoptosis (Waldman et al., 1996). Paradoxically, overexpression of p21 in liver (Wu et al., 1996) seems to have the same effect on mouse hepatocytes in that hepatocytes overexpressing p21 seem to undergo polyploidisation (Wu et al., 1996). In addition, pRb (the retinoblastoma protein), as part of the p21 regulatory pathway, has recently been implicated in preventing endoreduplication in human cells (Niculescu III et al., 1998). The exact role of p21 and the precise mechanisms involved in this process remain unknown.

Polyploidisation in mammalian liver

Although re-replication of DNA in the absence of mitosis to give rise to polyploid nuclei is something that in most cells does not occur, polyploidisation in the parenchymal cells of the livers of adult mammals has long been recognised. However, the physiological significance of this process is still a matter of speculation (see Chapter 3, section 3.3 for additional information) and the biochemical bases of polyploidisation remain a mystery. It is generally assumed that polyploidisation is a hormonally regulated, age-related process which varies according to the mammalian species under study (Medvedev, 1986). The patterns of polyploidisation in rat and human liver are similar but they differ from that of the mouse. In fact, the periods of polyploidisation in human liver are restricted to the periods of active growth and development and, contrary to the situation in mouse liver, polyploidisation of human hepatocytes at the end of the growth period does not attain as high levels as in the mouse (Kudryavtsev et al., 1993). In addition, unlike the situation in mouse liver, a rapid increase in the number of binucleate cells and accumulation of high ploidy levels in hepatocytes are not typ-

ical of the human liver during the postnatal liver growth period (Kudryavtsev et al., 1993). In rat the polyploidisation pattern of hepatocytes is progressive only until 12-14 months of age and does not continue into senescence. There are no further changes in the polyploidisation of rat hepatocytes between 14 and 27 months of age where the average DNA content corresponds to that of tetraploidy. Overall, the polyploidisation patterns of rat and human livers are very similar and differ significantly from the pattern followed by hepatocytes from the mouse liver, where the accumulation of hepatocytes with increasing ploidy continues even during senescence. The senescence period in mice (age between 14 and 28 months) also coincides with the accumulation of 8C, 16C and 32C nuclei. Even during the later stages of senescence (28-33 months of age) when the liver biomass starts to decline there is a continuing accumulation of highly polyploid 32C and 64C hepatocyte nuclei (Medvedev, 1986).

1.6.3 G1/S damage checkpoint

S phase delay

It has already been discussed in the Replication section 1.4 that cells that have been irradiated in S show a temporary inhibition of replication and that the degree and duration of the delay are dose-dependent. It has been observed in normal human diploid fibroblasts that the radiation dose-response curve is biphasic and that doses of 10 Gy and below inhibit uptake of tritiated thymidine more effectively than doses above 10 Gy (Rowley, 1998). If the incorporation of radioactivity into the newly synthesised DNA was measured in these cells 30 minutes after irradiation (5 and 20 Gy), a strong inhibition was observed in irradiated cells, suggesting that the inhibition occurs preferentially at the initiation of replication. It is possible that the biphasic nature of the dose-response curve arises from the differential radiosensitivity of initiation vs elongation of the daughter DNA molecule.

Is this suppression of initiation, elongation or both the result of a checkpoint? Mutations in the ATM gene (this gene is found mutated in AT patients), alters the shape of the radiation-response curve for inhibition of replication. In fact, it has been shown that in AT cells, inhibition of DNA replicon initiation does not occur. The existence of this mutant argues in favor of a checkpoint-mediated mechanism for the inhibition

of replicon initiation (Rowley, 1998).

As with G2 delay, S phase or replicon initiation delay in HeLa and V79 cells, is abolished by continuous caffeine treatment and postponed by finite treatments. Thus, elongation of daughter DNA continues after irradiation, as observed for AT cells. The mechanisms involved in this caffeine effect are not well understood (Rowley, 1998).

As with the G2 checkpoint, no mammalian cell mutant is known that is defective for the replicon initiation checkpoint only, and nothing is known of the consequences of this particular checkpoint defect. It has been speculated, however, that the role of replicon initiation delay is to allow repair of DNA lesions before replication is initiated. This repair would be relatively error-free as opposed to post-replication repair of chromatid breaks (see Replication section 1.2).

Painter has suggested that a single DNA DSB in a replicon cluster may result in the deformation of the chromatin structure in that domain and that this acts to signal the DNA synthesis delay (Painter, 1986), but the actual nature of this arrest still remains speculative. The molecular mechanisms responsible for this delay are not yet known either. In mammalian cells, the only gene known that is required for the S phase checkpoint control is ATM, but very little is known about this gene or its function, apart from the fact that p53 stabilisation in response to ionizing radiation in AT cells is impaired.

G1 delay

In normal cells there are at least two pathways that are used as regulation points where DNA damage leads to arrest. One of them, the G2/M checkpoint has been discussed in the Cell Cycle Control I section 1.5. Whereas the G2/M checkpoint is best understood in yeast, the G1 checkpoint is best understood in mammals.

Recent work has implicated the tumour suppressor gene p53 as a key player in the G1 cell cycle checkpoint in mammalian cells. Levels of p53 protein are elevated in response to DNA damage induced by a number of agents (Lees and Harlow, 1995). Tumour cells lacking p53, or having a dominant mutation in p53, are defective in their cell cycle progression delay at G1 in response to ionizing radiation. By restoring wild type p53 function in these cells the delay is also restored, and addition of dominant mutant

p53 to wild type cells prevents delay. The mechanism of p53 action is not completely understood, but at least it is known the chromosomal breaks lead to increased levels of p53. It appears that the point in G1 at which cells are delayed by the p53-dependent checkpoint control coincides with the restriction point, that is, the point in the cell cycle beyond which the cell commits to division and loses the potential to differentiate, or enter a state of quiescence. This is based on observations that the activities of cdk4 and cdk6 can be modulated by p53 (see below). These two cdks associate with D-type cyclins and they appear to be part of a restriction point since their expression influences the duration of G1 and the cellular dependence on mitogens (Rowley, 1998). In addition it has been shown that tyrosine phosphorylation of cdk4 is required to block cells in G1 after UV irradiation.

Apparently the p53-dependent delay in G1 following DNA damage can be abolished by caffeine, but the effective concentration seems to be cell-line-independent (Rowley, 1998). If this is true, it means that there is a caffeine susceptible checkpoint-control component that mediates delays in all three phases of interphase (see previous sections).

As explained and discussed in the Replication section 1.2, treatment of cells with cycloheximide, which inhibits cell progression, before cells are irradiated, results in an increase of cell survival with increasing time to release from the cycloheximide-induced arrest. This suggests that if cells are given time before progress into S phase, the cells are capable of repairing the damage. Surprisingly, loss of G1 checkpoint control does not seem to result in an increase of radiation induced killing. This paradox has an explanation in the fact that cells that fail to arrest in G1 go on to delay longer at subsequent checkpoints in S phase or G2, allowing for repair to take place then. A p53-deficient MCF-7 cell population has been shown to arrest at G2 with higher frequency (cells are arrested at G2 at 16 hours after 6.3 Gy) than the p53 wild-type population (Fan et al., 1995).

Failure to delay in G1 increases the frequency of gene amplification in irradiated cells (Rowley, 1998). Gene amplification occurs at a rate of 10^{-5} - 10^{-3} in cells without a functional p53-dependent checkpoint control and at an undetectable rate in normal diploid fibroblasts (Rowley, 1998). It has been proposed that failure to delay in G1 after DNA damage leaves insufficient time for repair of chromosome breaks. Such breaks may

then be replicated upon passage through S phase, by joining sister chromatids, with consequent bridge formation in mitosis, non-disjunction and the unequal segregation of specific genes. Repetition of this process leads to gene amplification. Therefore, repair in G1 is relatively error-free compared to repair in G2, when the break has already been replicated. Mice lacking both copies of p53 develop relatively normally, but they develop tumours by 6 months of age, which confirms the relationship between increased genomic instability and predisposition to cancer. p53 mutations are found in 50% of all known human tumours. A number of cancer-prone syndromes exist, i.e., Li-Fraumeni syndrome, Fanconi's anaemia, Werner's syndrome, Bloom's syndrome and ataxia-telangiectasia (AT)(also see Premature ageing section 1.1.5 and Chapter 6), that provide evidence that links genetic instability to DNA damage repair mechanisms, DNA damage recognition pathways and cell cycle control regulation. In particular, AT points directly towards a direct role of p53 in these processes. AT is caused by a recessive mutation that inactivates the feedback mechanisms that regulate DNA replication in irradiated cells. Cells from these patients are unable to induce p53 stabilisation in response to ionizing radiation. These results point towards a defect in the signal transduction pathway that involves p53 and that controls cell cycle arrest following DNA damage.

G1 delay is observed after irradiation (either UV or ionizing), treatment with topoisomerase II inhibitors, a DNA restriction enzyme, alkylating agents, depleted medium, heat or hypoxia. Thus, G1 delay seems to be induced by a wide variety of factors, including DNA damage. p53 in particular is induced by agents that either induce DNA damage directly (ionizing radiation) or initiate processes such as repair or replication, which lead to the formation of DNA strand breaks (Nelson and Kastan, 1994).

Most theories support the idea that the G1 arrest consist of two major components: a detector of DNA damage and an effector of progression arrest. The effector for the induction of the G1 delay in response to DNA damage is most likely to be a cdk. The detector remains unknown, but there are candidate proteins such as the Ku protein and the associated double-stranded DNA-dependent protein kinase (DNA-PK). Signal transduction factors may include p53 and also the ATM gene.

Various reports support the idea that levels of p53 protein increase after DNA damage

in a variety of cells (Abrahams et al., 1995; Reinke and Lozano, 1997; Poon and Hunter, 1998; Li et al., 1996; Nelson and Kastan, 1994; Lu and Lane, 1993; Dumaz et al., 1998; Maltzman and Czyzyk, 1984). The DNA damage-induced stabilisation of p53 seems to be brought about by blocking a ubiquitin-dependent p53 degradation pathway. p53 regulation is defective in AT cells, suggesting that ATM protein Kinase regulates p53 levels. A total of 10 phosphorylation sites have been identified in rat p53 (Rowley, 1998). MAP kinase, casein kinase I, casein kinase II (CKII) and cdk1 can phosphorylate p53. Mutation of the CKII site results in loss of growth suppression by p53, and both DNA-PK and CKII can promote tetramerisation of p53, a conformation required for DNA binding, although defects in DNA-PK do not seem to affect p53 stability, since p53 function is normal in SCID mouse fibroblasts (deficient in DNA-PK function). Phosphorylation of serine-12 by cdk1 modulates the nuclear localisation signal of p53 (Rowley, 1998).

Downstream effectors of p53 function are better characterised. p53 is a transcription factor, and its stabilisation results in the transcriptional activation of key regulatory genes, which in turn play a direct role in the arrest of cell cycle progression. One of the best understood p53-induced genes is p21. As mentioned before, p21 physically associates with cdk-cyclin complexes whereby inactivating the kinase activity of these cdk-cyclin complexes. The direct involvement of p53 in the transcriptional induction of p21, brought about by the existence of p53-responsive elements in the p21 promoter region, links p53 directly to the cell cycle. p53 is therefore a key 'connector' between DNA damage and inhibitory regulation of the cell cycle at the G1/S transition.

If the G1 delay provides time for repair of DNA damage, then a system to coordinate repair and progression seems likely to exist. As shown before, in normal mammalian cells p21 is found complexed with a cyclin, a cdk and most importantly in the context of DNA repair, with PCNA. PCNA, apart from being an essential component of the repair machinery (see Replication section 1.2) and being found at sites of replication, can also be found at putative sites of DNA repair following irradiation (Rowley, 1998). p21 directly blocks PCNA-dependent DNA synthesis and activation of polymerase- δ (Waga et al., 1994) and Li *et al.* showed that although p21 blocks replicative DNA synthesis, it allows PCNA to function in DNA repair (Li et al., 1994b). Each type of

p21 inhibitory function requires different domains in the p21 protein, i.e., inhibition of cdk activity by p21 required the amino-terminal, whereas inhibition of PCNA function required the carboxy-terminal residues of p21 (Chen et al., 1995). Like p21, GADD45 is induced by ionizing radiation in a p53-dependent manner and associates with PCNA (Rowley, 1998). When GADD45 was removed from an *in vitro* excision repair reaction, by immunodepletion, the rate of repair was decreased (Smith et al., 1994). However, the precise role of GADD45 is not known, but it is likely that it interacts with and inactivates a form of PCNA required for DNA replication, thus blocking entry to S phase while simultaneously stimulating repair (Rowley, 1998). GADD45 can be induced by UV or MMS, but not ionizing radiation, in the absence of a functional p53 protein (Rowley, 1998).

1.7 Aims

The aim of this thesis is to use histology, cytology and biochemistry to find a model that links cellular processes such as DNA repair, cell cycle control and cellular differentiation and senescence in order to explain the phenotype seen in the livers of ERCC1-deficient mice. In order to do so a number of different approaches have been brought together, from liver morphology, immunohistochemistry and immunocytochemistry studies, to *in vitro* biochemical studies of different UV-induced damage responses in primary and transformed fibroblasts. A subsidiary aim of this thesis is to investigate the possibility of the existence of a human disease related to a defect in ERCC1. The human disease chosen for this study has been the premature ageing syndrome Hutchinson-Gilford progeria. The ERCC1 status in the progeria fibroblasts as well as the response of progeria cells to DNA damage have been assessed.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Suppliers of laboratory reagents

Restriction endonucleases and other nucleic acid modifying enzymes

- Boehringer Mannheim PLC.
- GIBCO BRL Life Technologies.
- New England Biolabs Inc.
- Pharmacia LKB Biotechnology.

Standard laboratory reagents

- BDH Chemicals Ltd.
- Fisons Chemicals.
- GIBCO BRL Life Technologies.
- ICN Flow Ltd.
- Sigma Chemical Co.
- New England Biolabs Inc.

Reagents for cell culture

- GIBCO BRL Life Technologies.
- ICN Flow Ltd.
- Sera-lab Ltd.
- Sigma Chemical Co.

Radioactive reagents

- [α - 32 P] dCTP 3000 Ci/mmol, Amersham Redivue
- [*methyl* - 3 H] Thymidine 25 Ci/mmol, Amersham Life Science

Antibiotics

- Penicillin G, Sigma Chemical Co.
- Streptomycin, Sigma Chemical Co.

Primer synthesis

- Perkin-Elmer Ltd.
- Oswel DNA service

Antisera*Primary antibodies*

1. Anti ERCC1 antibodies. These antisera were kindly donated by Dr. Richard Wood, ICRF, Clare Hall laboratories.
 - RW09: rabbit polyclonal against a synthetic ERCC1 peptide (aa282-aa294)(R. Wood personal communication).
 - RW017: rabbit polyclonal against the whole human protein expressed in *E. coli* (R. Wood personal communication).

2. XPF(ERCC4)antibody. This antiserum was kindly donated by Dr. Richard Wood, Clare Hall laboratories.
 - RA1: rabbit polyclonal raised against the C-terminal half of XPF (expressed in *E. coli*)(R. Wood, personal communication).
3. Anti p53 antibody
 - Ab-7: sheep polyclonal raised against human p53 (expressed in *E. coli*)(Oncogene Research Products)
4. Anti ligase antibodies were kindly provided by Dr. Tomas Lindahl and co-workers, ICRF, Clare Hall laboratories.
 - TL6: rabbit polyclonal raised against a conserved peptide in the C-terminus of Ligase I (Tomkinson et al., 1990).
 - TL18: rabbit polyclonal antibody raised against a peptide in the C-terminus of Ligase IV (Tomas Lindahl, personal communication).
 - TL25: rabbit polyclonal antibody against recombinant full length human DNA ligase III (Tomas Lindahl, personal communication).
5. Vimentin antibody. Vimentin is an intermediate filament protein (MW 57 kDa) of mesenchymal cells. It is used as a fibroblast marker.
 - Vim3B4: mouse monoclonal against the whole purified bovine protein (Bradsure Biologicals Ltd.).
6. Anti BrdU antibody,
 - MAS250: rat monoclonal; clone BU1/75 (Harlan, SeraLab)
7. Anti Ki67 antibody,
 - MIB1: rabbit polyclonal; clone MIB-1 (Immunotech)
8. Centromeric staining antibodies: These 3 antibodies were kindly donated by Prof. William Earnshaw, Edinburgh University.

- NR: Antiserum obtained from a patient with scleroderma. NR recognises the centromeric proteins Cenp A, B and C. NR stands for the name of the rheumatologist who supplied the serum, Naomi Rothfield (Earnshaw and Rothfield, 1985).
- Biotin-conjugated anti-human IgG.
- Streptavidin-conjugated Texas Red tertiary antibody.

Secondary antibodies

1. Biotinylated rabbit anti-sheep IgG: Calbiochem (Oncogene Research Products)
2. Biotinylated goat anti-rabbit IgG: Calbiochem (Oncogene Research Products)
3. Biotinylated swine anti-rabbit IgG: DAKO Ltd.

Tertiary reagents

1. Streptavidin: as part of the StreptABComplex/HRP kit (DAKO Ltd.)
2. Biotinylated Horseradish Peroxidase: as part of the StreptABComplex/HRP kit (DAKO Ltd.)

2.1.2 Standard buffers and solutions

- 20× SSC: 3 M NaCl; 0.3 M tri-sodium citrate, pH 7.0
- 10× TBE: 0.9 M Tris-HCl; 0.9 M boric acid, 20 mM EDTA, pH 8.0
- TE: 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0
- PBS: 8 mM K₂HPO₄, 1.5 mM KH₂PO₄, 150 mM NaCl
- TBS: 12.5 mM Tris.HCl, pH7.6, 150 mM NaCl
- TBST: 12.5 mM Tris.HCl, pH7.6, 150 mM NaCl, 0.01-0.5% (v/v) Tween 20
- 10× MOPS: 200 mM MOPS, 50 mM sodium acetate, 10 mM EDTA, pH 7.0
- Lysis Buffer (Protein Extraction): 125 mM Tris pH7, 33 mM DTT, 2% SDS, 2 mM PMSF, 2mM EDTA, pH 8.

- Denaturing Buffer: 0.5 M NaOH, 1.5 M NaCl
- Neutralising buffer: 3 M NaCl, 0.5 M Tris.HCl, pH 7.0
- Scintillation Fluid: 2.5 ltr Toluene, 15 g PPO (2,5-Diphenyloxazole), 0.4 g POPOP (1,4-bis[5-Phenyl-2-oxazolyl]benzene; 2,2'-p-Phenylene-bis[phenyloxazole])
- PCA: 25 parts redistilled phenol, 24 parts chloroform, 1 part isoamyl alcohol
- CA: 24 parts chloroform, 1 part isoamyl alcohol
- Tail buffer: 300 M sodium acetate, 10 mM Tris-HCl, 1 mM EDTA, 1% SDS
- 1×Protein electrophoresis buffer: 20 mM Tris, 192 mM Glycine, 0.1% SDS, pH 8.3
- 10×PCR buffer: 50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl pH 8.3, 0.01% (w/v) gelatin, 0.45% (v/v) Triton X-100, 0.45% (v/v) Tween 20

2.1.3 Cell culture media and cell lines

Fibroblast culture medium

- Normal fibroblast culture medium: 10% (v/v) Foetal Calf Serum (FCS) or 20% (v/v) FCS in the case of AG08466 primary fibroblasts, 1 × non-essential amino acids, 25 U/ml penicillin, 1 mM sodium pyruvate, 25 µg/ml streptomycin.
- GMEM Serum-free fibroblast culture medium: 1× non-essential amino acids, 25 U/ml penicillin, 1 mM sodium pyruvate, 25 µg/ml streptomycin, in GMEM.

Freezing medium for long term storage of cells

This medium consisted of the appropriate supplemented culture medium with additional DMSO and FCS to final concentrations of 10% (v/v) and 20% (v/v) respectively.

Cell lines

- 46BR.1G1: SV40 transformed human fibroblast cell line with aberrant DNA ligase I activity (Webster et al., 1992).

- MRC5V1: SV40 transformed wild-type human fibroblast cell line (Huschtscha and Holliday, 1983).
- PF20: Spontaneously immortalised wild type mouse embryonic fibroblast cell line (Melton et al., 1998).
- PF24: Spontaneously immortalised ERCC1-deficient/HPRT-deficient mouse embryonic fibroblast cell line (Melton et al., 1998).
- CHO9: Immortalised wild type Chinese hamster ovary cell line, which is a subclone of the CHO-KK cell line (Burki et al., 1980). The cells used in the replication experiments carried out in Cambridge (see Methods below), were kindly donated by S. Squires and R. Johnson, Zoology Dept., Cambridge University. The cells used in Edinburgh were kindly donated by Richard Wood, ICRF, Clare Hall.
- CHO 43-3B: Immortalised ERCC1-deficient Chinese hamster ovary cell line, derived from the parental CHO9 cell line (Wood et al., 1982). The cells used in the replication experiments carried out in Cambridge (see Methods below), were kindly donated by S. Squires and R. Johnson, Zoology Dept., Cambridge University. The cells used in Edinburgh were kindly donated by Richard Wood, ICRF, Clare Hall.
- CHO AA8: Immortalised wild type Chinese hamster ovary cell line (Thompson et al., 1980). Kindly donated by S. Squires and R. Johnson, Zoology Dept., Cambridge University.
- CHO UV5: Immortalised ERCC2-deficient Chinese hamster ovary cell line, derived from the parental CHO AA8 cell line (Thompson et al., 1981). Kindly donated by S. Squires and R. Johnson, Zoology Dept., Cambridge University.
- A2780: Human ovarian adenocarcinoma cell line. Epithelial morphology (Behrens et al., 1987).
- AG08466: Human primary skin fibroblast isolated from a patient diagnosed with Hutchinson-Gilford syndrome. Obtained from the N.I.A. ageing cell culture

repository, Coriell Institute for Medical Research, 401 Haddon Ave., Camden, NJ 08103.

2.1.4 Miscellaneous

- Proteinase K (PK): 5 mg/ml in d.H₂O (Boehringer Mannheim), stored at 4°C.
- Trypsin: 0.025% (w/v) trypsin (ICN Flow, Cat No. 043-5090), 1 mM EDTA, 1%(v/v) chicken serum (ICN Flow Cat No.29-501-49) in 1× PBS.
- 50× DAB (3,3' Diaminobenzidine): 125 mg in 5 ml d.H₂O, aliquoted in 100 µl aliquots and stored at -20°C.

2.2 Methods

2.2.1 Isolation of primary embryonic fibroblasts

Dissection of conceptuses

Plugged females were killed on the required day of embryonic development, and the uteri were dissected out of the body cavity. The muscle layer of the uterus was removed, along with the Reichert's membrane and placenta, to leave the embryo and visceral yolk sac. Embryos were incubated in trypsin and disaggregated for embryonic fibroblast isolation.

Isolation of mouse embryonic fibroblast cultures

Primary fibroblasts were isolated from E10.5 or E11.5 embryos. Following dissection, embryos were incubated in 0.5 ml trypsin for 30 minutes at 37°C. 15 mls of medium were added, the embryo mechanically disaggregated with a pipette, and cells plated out into 90 mm dishes.

Growth of mammalian fibroblasts

Cells were maintained in supplemented GMEM at 37°C in a 5% CO₂ atmosphere in a humidified tissue culture incubator (Forma Scientific). To passage cells, the medium

was aspirated, the bottom of the flask rinsed with 1-2 mls of trypsin, then a further 1-2 mls of trypsin added and incubated at 37°C until the cells had become detached. Thereupon 5 volumes of medium were added, and the cells pelleted by 5 minutes centrifugation at 1,300 rpm. The supernatant was discarded, and the cells resuspended in an appropriate volume of medium, and an aliquot transferred into a 90 mm dish. For long term storage, cells were kept in liquid nitrogen. Cultures to be frozen were trypsinised, spun down, the supernatant removed, and cells resuspended in ice cold freezing medium. 1 ml aliquots were placed at -20°C for 2 hours, then transferred to -70°C overnight, before removal into liquid nitrogen. Aliquots were thawed rapidly at 37°C and diluted in 9 mls of medium, before being pelleted by centrifugation at 1,300 rpm for 5 minutes. The supernatant was discarded, the cells resuspended in an appropriate volume of medium, and transferred into a flask.

2.2.2 Preparation of genomic DNA from cells

High molecular weight cell genomic DNA, suitable for PCR amplification and Southern analysis was prepared from mouse tail samples, and fibroblasts by the same method. Cultured cells were harvested by scraping in ice cold PBS, and pelleted by centrifugation at 1,300 rpm for 5 minutes. Cell pellets were digested overnight at 37°C in 700 μ l tail buffer supplemented with proteinase K to a final concentration of 280 μ g/ml. The supernatant was extracted twice with 700 μ l PCA and vigorous shaking, and subsequently once with 700 μ l CA to remove traces of phenol. Thereupon the DNA was precipitated for 10 minutes at room temperature by addition of 25 μ l 3.0 M sodium acetate pH 5.0 and 700 μ l of isopropanol. Following 10 minutes centrifugation, the nucleic acid pellet was washed twice with 70% (v/v) ethanol, vacuum dried, resuspended in 200 μ l sterile distilled water and stored either at 4°C in the short term or at -20°C for longer periods.

2.2.3 Preparation of RNA from cells and mouse tissues

The following method is adapted from Chomczynski and Sacchi, 1987. Fresh cells or tissue were placed in a sterile polycarbonate centrifuge tube containing up to 7 ml of RNazol solution (Biogenesis Ltd.) and homogenised. Subsequently, 0.1 volumes of

chloroform was added, the tube was shaken vigorously for 30 seconds and stored on ice for 5 minutes and then centrifuged for 15 minutes at 10,000 rpm in an HB4 rotor pre-cooled to 4°C. The aqueous phase was transferred to a fresh centrifuge tube and RNA precipitated by the addition of 1 volume of isopropanol. The sample was stored at 4°C for at least 15 minutes before a 15 minute centrifugation as above. The RNA pellet was washed in 75% ethanol, centrifuged for 8 minutes at 5,000 rpm and resuspended in sterile d.H₂O (Chomczynski and Sacchi, 1987). RNA was generally stored at -70°C.

2.2.4 Quantification of nucleic acids

Estimation of DNA concentration

The DNA sample was diluted in 1 ml of distilled water and the OD of absorbance at wavelengths 260nm and 280nm was measured by a spectrophotometer (Perkin-Elmer, Lambda 15, UV/VIS Spectrophotometer). An OD_{260nm} value of 1.0 represents a concentration of 50 mg/ml for DNA. The ratio OD_{260nm}/OD_{280nm} provides an estimate for the purity of the nucleic acid. A value in excess of 1.8 indicates a pure preparation of DNA.

Estimation of RNA concentration

The RNA sample was diluted in TE and the OD of absorbance at wavelengths 260nm and 280nm was measured by a spectrophotometer (Perkin-Elmer, Lambda 15, UV/VIS Spectrophotometer). An OD_{260nm} value of 1.0 represents a concentration of 40 mg/ml for RNA. The ratio OD_{260nm}/OD_{280nm} provides an estimate for the purity of the nucleic acid. A value in excess of 2.0 indicates a pure preparation of RNA.

2.2.5 DNA manipulation

Digestion of DNA with restriction endonucleases

Most DNA restrictions were performed using BRL, Boehringer Mannheim or New England Biolabs enzymes and buffers. DNA was digested with approximately 2–5 U of restriction endonuclease per μ g of DNA using buffer and temperature conditions recommended by the manufacturer. For double digests involving enzymes with different

recommended buffers, the buffers were checked individually in double digests to determine which gave most efficient digestion. If the optimal digestion conditions varied for buffers, the DNA was digested with enzyme at lower salt concentration, the enzyme reaction stopped by heat denaturation or phenol extraction, the DNA precipitated and dissolved in a buffer appropriate for digestion by the next enzyme. Digestion reactions were terminated by addition of DNA sample buffer then heated for 10 minutes at 65°C prior to electrophoresis.

2.2.6 Electrophoresis of nucleic acids

Electrophoresis of DNA in agarose gels

DNA was separated in 0.7-2.0% (w/v) electrophoresis grade agarose with 0.5 µg/ml ethidium bromide in 1× TBE buffer (for Southern blotting and routine analytical gels). Prior to loading, DNA samples were mixed with 1/5 volume of 5× sample buffer (20% (v/v) glycerol, 100 mM EDTA, 0.1% bromophenol blue). Electrophoresis was carried out horizontally across a potential difference of 1-10 V/cm. Bacteriophage λ DNA cut with HindIII and ϕX174 DNA cut with HaeIII were used as size markers. DNA was visualised by short wave UV illumination. Preparative gels were not exposed to short wave UV as this can result in thymidine dimerisation. Instead, the DNA in preparative gels was visualised by long wave UV from a hand held lamp.

Electrophoresis of RNA in agarose gels

RNA samples were electrophoresed through 1.4% (w/v) agarose gels made with 1× MOPS, 0.66 M formaldehyde and 0.5 µg/ml ethidium bromide. Samples were prepared by the addition of 30 µg of total RNA (in 20 µl d.H₂O) to an equal volume of FSB (100 µl 10× MOPS, 200 µl formamide, 120 µl de-ionised 37% (w/v) formaldehyde) and one quarter volume of standard sample buffer (20% glycerol, 100 mM EDTA, 0.1% bromophenol blue) was also added and the sample heated for 5 minutes at 65°C then cooled on ice prior to electrophoresis for 3-4 hours at 100 V.

Recovery of DNA from agarose gels

DNA was electrophoresed using low melting point agarose (LMPA) in $1\times$ TBE, 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide, the desired fragment was visualised by long wave UV illumination. The band of interest was excised and recovered from the agarose by phenol extraction. The gel slice volume was estimated then 0.5 volumes of TE, 1 μl glycogen (Boehringer Mannheim) and 5 μl 20% SDS was added to the gel slice and heated to 70°C until the agarose melted (approx. 5 minutes). Liquified agarose was extracted three times with 1 volume of tris buffered phenol (pH 8.0) and once with 1 volume of chloroform. The aqueous phase was retained and DNA precipitated by the addition of 0.1 volume 3 M sodium acetate and 1 volume of isopropanol. DNA was pelleted by a 10 minute centrifugation at 13 Krpm and the DNA pellet resuspended in $\text{d.H}_2\text{O}$.

2.2.7 Transfer of nucleic acids

Transfer of DNA from agarose gels to membranes

The Southern blot presented in this Thesis has been carried out by James Selfridge. Genomic DNA was digested with the appropriate restriction enzyme in a total volume of 50-100 μl overnight at 37°C and separated according to size on a 0.7-1% agarose gel (w/v) in $1\times$ TBE and 0.5 mg/ml ethidium bromide. Routinely, genomic DNA was electrophoresed for approx. 800Vhours then photographed. The gel was then soaked in denaturation buffer (0.5 M NaOH, 1.5 M NaCl) with gentle agitation for 15 minutes. The DNA was transferred to Genescreen Plus nylon membrane (Du Pont; Stevenage, U.K.) by capillary action using denaturation buffer as the transfer medium (Reed and Mann, 1985). Transfer was performed for 12-24 hours then the membrane was neutralised and adherent agarose washed off by incubating for 10 min in 3 M NaCl, 0.5 M Tris-HCl pH 7.0 on a rocking platform. Filters were air dried before use.

RNA transfer

Following electrophoresis, gels were washed three times for 20 minutes in $10\times\text{SSC}$ at room temperature to remove formaldehyde and ethidium bromide. Transfer conditions were similar to DNA except that $10\times\text{SSC}$ was used as the transfer buffer. On com-

pletion of transfer the membrane was washed in $2\times$ SSC for 10 minutes then baked at 80°C for 2 hours.

2.2.8 Nucleic acid hybridisation

Labelling DNA probes

DNA labelled to high activities was obtained using the randomly primed DNA labelling method (Feinberg and Vogelstein, 1983). DNA probes were denatured, annealed to random hexamer primers and extended by the Klenow fragment of *E.coli* DNA polymerase I in the presence of $[\alpha -^{32}\text{P}]\text{dCTP}$. Oligonucleotide labelling buffer (OLB) containing nucleotides and random primers is required for this method. It is made by mixing 50 ml Solution A (1.25 M Tris-HCl pH 8.0, 0.125 M MgCl_2 , 25 mM β -mercaptoethanol, 0.5 mM each of dGTP, dTTP and dATP), 125 ml Solution B (2 M HEPES buffer adjusted to pH 6.6 with NaOH) and 75 ml Solution C (random hexanucleotides $\text{OD}_{260\text{nm}} = 90$ in TE). Probes were labelled as follows: 100 ng of double-stranded DNA was made up to $32\mu\text{l}$ with $\text{d.H}_2\text{O}$ then denatured by boiling for 5 minutes and allowed to cool at 37°C for 10 minutes. The following reagents were then added: $10\mu\text{l}$ of OLB, $2\mu\text{l}$ of 10 mg/ml BSA, $5\mu\text{l}$ of $[\alpha -^{32}\text{P}]\text{dCTP}$ (3000Ci/mmol) and 10 units of Klenow enzyme. The reaction was incubated overnight at room temperature.

DNA and RNA hybridisations

Pre-hybridisations were performed in a hybridisation oven (Hybaid Limited) in a total volume not exceeding 30 ml of prehybridisation buffer ($6\times$ SSC, 1% SDS, 10% (w/v) dextran sulphate, 100 mg/ml of denatured herring sperm DNA). Blots were prehybridised for a minimum of 2 hours at 65°C for Southern transfer and at 60°C for northern transfer. DNA probes were heat denatured in $300\mu\text{l}$ of 10 mg/ml herring sperm DNA and added to the prehybridisation solution after purifying them through a Nick Column, Sephadex G-50, DNA grade (Pharmacia Biotech). Following hybridisation overnight at 65°C , the hybridisation solution was discarded and the blots were rinsed for 5 minutes in $2\times$ SSC at room temperature, followed by a 30 minute wash in $2\times$ SSC, 1% (w/v) SDS at 65°C then a 30 minute wash in $2\times$ SSC, 1% (w/v) SDS at 65°C . Membranes were sealed in a plastic bag and autoradiographed at -80°C , typically

overnight. Northern blots were hybridised and washed as above but at 60°C.

Autoradiography

Autoradiography was performed using Cronex X-ray film (DuPont) in a cassette with intensifying screens (Cronex Lightning Plus; Du Pont). Cassettes were exposed at -70°C to facilitate fluorographic enhancement.

Stripping probes from membranes

Radiolabelled probes were stripped from Southern blots by boiling in 0.1× SSC, 1% (w/v) SDS for 10 minutes with gentle shaking. The solution was decanted from the membrane, and the boiling procedure was repeated twice. Blots were autoradiographed to confirm that probe stripping was complete. The membrane was then incubated in pre-hybridisation solution and hybridised as above. Northern blots were stripped by washing them 4 times for 5 minutes in boiling 0.01× SSC, 0.01% (w/v) SDS.

2.2.9 DNA sequencing

The cDNA sequencing procedure presented in this Thesis was carried out by Dr. Nicola Redhead.

Reverse Transcription of RNA

Typically, 10µg of RNA in a volume of 10µl were used per reaction as follows (Table 2.1).

The reaction mix was incubated at 43°C for 1 hour and after that time an additional 0.5µl RNasin and 1.5µl of RTase were added per reaction. The samples were incubated at the same temperature for a further 1 hour.

Samples were stored at 4°C after addition of 200µl TE.

- 10×RT buffer: 500 mM Tris.HCl pH 8.3, 60 mM MgCl₂, 400 mM KCl, 10 mM DTT. Stored at -20°C.

<i>Reagents</i>	<i>+Rtase</i>	<i>–Rtase</i>
RNA	10 μ l	10 μ l
d.H ₂ O	3.5 μ l	5 μ l
10 \times RT buffer (see below)	2 μ l	2 μ l
RNasin (40 U/ μ l)	0.5 μ l	0.5 μ l
25 mM dNTP	1 μ l	1 μ l
Hexameres (0.1 μ g/ μ l)	1 μ l	1 μ l
dT _{12–18} (50 μ g/ml)	0.5 μ l	0.5 μ l
RTase (20 U/ μ l)	1.5 μ l	–

Table 2.1: Reverse Transcription Reaction. Typical ingredients.

PCR amplification of cDNA's

20 μ l of cDNA prepared as described in the previous section were used for amplification by the PCR method as follows. All PCR reactions were carried out in 50 μ l volumes in 1 \times PCR buffer supplemented with EDTA and dNTPs, to a final concentration of 0.4 mM and 0.1 mM respectively, and 2.5 units of Taq DNA polymerase. Approximately 100ng of DNA was used per reaction, along with 1 ng of each primer (see Table 2.2). Cycle conditions varied for different primers pairs, but denaturation was generally carried out at 94°C for 1 minute, followed by 1 minute at an appropriate annealing temperature, and synthesis at 72°C for 1 to 2 minutes. 35–40 cycles were carried out for all PCR reactions. All experiments were carried out using an Omnigene thermal cycler (Hybaid Ltd.).

PCR conditions for the amplification of ERCC1 cDNA:

- 94°C for 1 minute
- 68°C for 1 minute
- 72°C for 1 minute

Repeat for 35 cycles.

The different PCR products obtained from the above PCR reactions were electrophoresed in a LMPA gel, the cDNA bands were extracted as explained elsewhere (see Recovery of DNA from agarose gels), and the cDNA's were subsequently sequenced (see below).

cDNA sequencing reactions

Dye terminator cycle DNA sequencing was performed using an ABI PRISM dye terminator cycle sequencing reaction ready kit (Perkin-Elmer Corporation), on an Omnigene thermal cycler (Hybaid Ltd.) and extension products separated on an ABI automated sequencer (Perkin-Elmer Corporation).

Approximately 0.4 μ g of cDNA template was mixed with 3.2 pmoles of an appropriate primer and 8 μ l of terminator ready reaction mix in a final volume of 20 μ l. The mixture was overlaid with a drop of mineral oil, and subjected to thermal cycling (25 cycles) as follows:

- 96°C for 30 seconds
- 50°C for 15 seconds
- 60°C for 4 minutes

Extension products were purified away from unincorporated terminators by addition of 2.0 μ l 3 M sodium acetate pH 4.8, 50 μ l absolute ethanol, and incubation on ice for 10 minutes followed by 15 minutes centrifugation.

The resulting pellet was washed with 70% (v/v) ethanol, vacuum dried, and stored dry at -20°C.

<i>Name</i>	<i>Sequence (5'→3')(Exon)</i>	<i>F/R</i>	<i>Primer combination</i>
C8438	CCCTTGAGGCTCCAAGACCAGCAGG (E1)	F	C8438-C8439
A9568	GATGGACCCTGGGAAGGACAAAGAGG (E2)	F	A9568-A9570
C8439	CCCTTGAGGCTCCAAGACCAGCAGG (E2)	R	C8439-C8438
A9569	CCGTACTGAAGTTCGTGCGCAACG (E4)	F	A9569-A9570&A9571
A9570	GCAAGGCGAAGTTCTTCCCCAGG (E5)	R	A9570-A9568&A9569
A9571	TCATCAGGGTACTTTCAAGAAGGG (E10)	R	A9571-A9569

Table 2.2: Primers used in the PCR reaction for the amplification of the human ERCC1 cDNA sequence. The polarity of the primer is represented by the letters F (forward) and R (reverse). The primer combination(s) each primer has been used in the PCR reaction is also shown.

Sequence analysis

Sequence analysis was carried out on a UNIX mainframe computer using the Genetics Computer Group (GCG) package of programs, version 9. Alignment of nucleotide sequences was performed using the Bestfit, Lineup, and Pileup programs.

2.2.10 PCR genotyping of mice

In order to determine the genotype of an individual, PCR reactions were performed on genomic DNA samples extracted from tail. Cycle conditions employed for different primer pairs are outlined below (Table 2.3) 20 μ l of each reaction was subjected to agarose gel electrophoresis, and products visualised by ultra-violet illumination.

2.2.11 Immunoassays

Immunoblotting

All proteins were detected by immunoblotting with crude liver or cell homogenates in conjunction with the appropriate antibody. Homogenates were prepared as follows: livers were dissected cleanly and homogenised in lysis buffer for protein extraction. Cells were scraped off their dishes and washed in PBS/0.01 M PMSF before homogenising in a sufficient volume of the same buffer. Total protein content was determined by the bicinchoninic acid method (BCA assay, Pierce). Samples were stored at -20°C prior to use. Homogenates and cell samples were boiled for 5 minutes with 1/2 volume of sample buffer before separation by electrophoresis in 1 \times electrophoresis buffer, using 8 or 12% SDS PAGE with a 3.3% stacking gel. NEB broad range pre-stained markers were run with all gels to assess separation and electrotransfer efficiency. Proteins were electroblotted from acrylamide gels onto PVDF membrane (Immobilon P, Millipore) using a BioRad Transblot SD semi dry electroblotter at 12V for 2 hours. Membranes were blocked overnight at 4°C with 3–10% dried milk in TBST (0.01–0.5%(v/v) Tween 20) (Table 2.4), the blocking solution was discarded and incubated with the appropriate primary antiserum diluted optimally in TBST or dried milk/TBST for 1 hour at room temperature (Table 2.4). Membranes were washed twice for 10 minutes in TBST then the secondary antiserum added (Table 2.4) and incubated for 1 hour. Membranes were

<i>Name</i>	<i>Sequence(5'→ 3')</i>	<i>Description</i>	<i>PCR Conditions</i>
033M	CCCGTGTTGAAGTTTGTGCG	Ercc1 exon 4 (McWhir et al., 1993b)	94°C 1', 68°C 1', 72°C 1' for 35 cycles
035M	CGAAGGGCGAAGTTCTTCCC	Ercc1 exon 5 (McWhir et al., 1993a)	
M4956	GGTTCGAAATGCCGACCAAGCG	Ercc1 neo cassette (D. Melton, personal communication)	
C1045	CAAAGAGCGTTGGGCATGTG	p53 intron 7; A. Clarke, personal communication	94°C 1', 62°C 1', 72°C 1' for 35 cycles
C1046	GTGGTGGTACCTTATGAGCC	p53 exon 6; A. Clarke, personal communication	
C1047	CATCGCCTTCTATCGCCTTC	p53 neo cassette; A. Clarke, personal communication	

Table 2.3: ERCC1 and p53 genotyping primers description

washed twice for 10 minutes in TBST, and then incubated in AB Complex (DAKO Ltd.) for 30 minutes at room temperature. Membranes were developed using a chromogenic method with DAB (see below, Immunohistochemistry).

Immunohistochemistry

Most of the BrdU- and all of the Ki67-staining was carried out in the immunohistochemistry section of the Pathology Department, Edinburgh University.

Livers were removed and immersion fixed for 24 hours in Methacarn (Methanol: Chloroform: Acetic Acid in a 4:2:1 ratio) followed by storage in 70% Ethanol. Livers were then dehydrated with alcohols and impregnated in wax during a 7 hour processing cycle. 3 μ m sections were mounted on slides which had been coated with poly-L-Lysine (PLL).

Mounted sections were typically immunostained by the standard ABC method. Briefly, sections were dewaxed in xylene and rehydrated in descending grades of alcohol. For the BrdU antibody it was necessary to render the cells permeable by incubating the slides in 1N HCl at 60°C for 1 hour. For Ki67 (MIB1) it was necessary to retrieve the antigen by microwaving the sections in citrate buffer (3.10 g citric acid/ litre of d.H₂O) for 5 minutes 3 times at high power. After either permeabilisation or antigen retrieval, the slides were washed in running water and subsequently rinsed in PBS for 5 minutes. The sections were subsequently blocked for endogenous peroxidase activity in 3% Hydrogen Peroxide for 10 minutes at room temperature and incubated a 1:5 dilution of Normal Goat Serum (SAPU) or Normal Rabbit Serum (SAPU) for 10 minutes. After this, the samples were incubated in an appropriate dilution of the primary antibody in Normal Serum in TBS for 30 minutes at room temperature, washed and incubated for 30 minutes in biotinylated secondary antibody diluted optimally in Normal Serum in TBS (Table 2.5).

After washing the secondary antibody off, the slides were incubated in AB Complex (Streptavidin/Biotin, DAKO Ltd., in DAB/ABC buffer, see below) for 30 minutes at room temperature. AB Complex was washed and the positive nuclei were visualised in DAB solution (see below) containing 100 μ l stock DAB, 100 μ l of 3% Hydrogen Peroxide

<i>Antibody</i>	<i>Blocking Solution</i>	<i>Primary Ab Dilution</i>	<i>Secondary Ab Dilution</i>	<i>Antibodies Solution</i>	<i>Washes</i>
Ab-7	3%M ¹ /TBST(0.1%T ²)	1:2,500	1/50,000	0.5%M ¹ /TBST(0.1%T ²)	TBST(0.1%T ²)
RW09	10%M/TBST(0.025%T)	1:200	1/7,500	10%M/TBST(0.025%T)	TBST(0.025%T)
RW017	10%M/TBST(0.01%T)	1:2,500	1/7,500	10%M/TBST(0.01%T)	TBST(0.01%T)
RA1	10%M/TBST(0.01%T)	1:1,500	1/7,500	10%M/TBST(0.01%T)	TBST(0.01%T)
TL6	3%M/TBST(0.05%T)	1:500	1/50,000	TBST(0.05%T)	TBST(0.05%T)
TL25	3%M/TBST(0.5%T)	1:500	1/50,000	TBST(0.5%T)	TBST(0.5%T)
TL18	3%M/TBST(0.01%T)	1:500	1/50,000	TBST(0.01%T)	TBST(0.01%T)
Vim3B4	3%M/TBST(0.05%T)	1:500	1/50,000	TBST(0.05%T)	TBST(0.05%T)

Table 2.4: Summary of the conditions used for the different Westerns.¹: 99% fat-free powder milk; ²: Tween

<i>Antibody</i>	<i>1st Ab Dilution</i>	<i>2nd Ab Dilution</i>	<i>Ab Solution</i>
BrdU(MAS250)	1:50	1:50	1:5 Normal Goat Serum in TBS
Ki67(MIB1)	1:100	1:400	1:5 Normal Rabbit Serum in TBS

Table 2.5: Immunohistochemistry conditions for BrdU and Ki67 staining of paraffin-embedded liver sections.

per 5 ml DAB/ABC buffer. This results in a final DAB concentration of 0.5 mg/ml .

Finally, the slides were counterstained lightly in Haematoxylin and Scott's Tap water (see below), dehydrated and mounted.

- DAB/ABC buffer: 50 mM Tris, 0.04N HCl
- Scott's Tap Water: 1% (w/v) KHCO_4 , 10% (w/v) MgSO_4 .
- Harris Haematoxylin (Sigma diagnostics): acidified before use with 16 ml of Glacial Acetic Acid per 350 ml stain.

Controls were carried out using preimmune serum in place of primary antibody.

Centromeric Staining of Hepatocytes

Livers were obtained from wild type and ERCC1-deficient mice (18–20 days of age). After dissection, the fresh livers were gently pressed onto a glass coverslip. This method allows single hepatocytes to detach from the liver in a non-disruptive manner so that their nuclei remain intact. Liver cells prepared in this way were allowed to air dry for 5 minutes and they were then fixed in 4% paraformaldehyde/PBS (see below). After washing in PBS the cells were permeabilised in KB buffer/0.1% Triton (see below), washed twice in PBS without Triton and incubated for 30 minutes at room temperature in the primary antibody diluted optimally (NR antibody, 1:1000 in PBS/0.1% Triton/0.1% Sodium Azide). The coverslips were washed again twice in PBS/1% Triton/0.1% Sodium Azide and subsequently incubated in a 1:1000 dilution of the biotinylated secondary antibody (anti-human IgG) in the same solution for 30 minutes at room temperature. After this, they were washed again and incubated for a further 30 minutes in the tertiary antibody solution (Streptavidin/Texas Red-complex, 1:1000

dilution in PBS/0.1% Triton/0.1% Sodium Azide) at room temperature. Finally, the cells were washed and stained with DAPI (0.5 $\mu\text{g}/\text{ml}$ in PBS) for 5 minutes, mounted on slides with Vectashield mounting medium and sealed with nail varnish.

Red dots within the nucleus of the hepatocytes corresponding to the centromeres in the chromosomes were scored using a fluorescence microscope (Axioplan 2, Zeiss) using a 100 \times /NA 1.4 oil immersion objective lens. The images presented in this thesis were acquired using a Sedat/Agard deconvolution microscope made by Applied Precision Inc. with an Olympus IX70 inverted microscope, a 100 \times /NA 1.4 oil immersion objective lens and a PXL CCD camera from Photometrics. Deconvolution is a computational method that compensates for the effect of out of focus light, resulting from the use of fluorescence microscopes, by re-assigning the light to its correct point of origin in a three-dimensional stack of images (Davis, 1999). This process was performed using DeltaVision software running on a Silicon Graphics computer. All manipulations performed with this microscope were carried out by Prof. William Earnshaw, ICMB, Edinburgh University. All pictures were processed using the Adobe PhotoShop or Powerpoint packages.

Buffers:

- 10 \times PBS-azide: 100 mM NaPO_4 pH 7.4, 1.5 M NaCl, 10 mM EGTA, 0.1 M NaN_3 .
- 10 \times KB: 100 mM tris.HCl pH 7.7, 1.5 M NaCl, 1% BSA.
- 1% Triton X-100: Made up in d. H_2O .
- 4% CH_2O in D-PBS: Made from paraformaldehyde. Dissolved by refluxing, then filtered through a 2 μm filter.

2.2.12 Replication Assays

Detailed Biochemical Assays: Repair/Stalling Experiments

All the experiments described in this section were carried out in the University of Cambridge, Zoology Dept., under the supervision of Dr. Robert T. Johnson and Dr. Shoshana Squires.

Cells were plated out at the desired density (2×10^5 cells/90 mm dish) on a Friday and left to settle down for 5-8 hours. At the end of the day [^{14}C] TdR was added to a final concentration of $0.1 \mu\text{Ci/ml}$ and left to label during the weekend until Tuesday.

[^{14}C] TdR medium was removed and the cells were allowed to grow in unlabelled medium for 16 hours. [^3H] TdR medium (to a final concentration of $1.0 \mu\text{Ci/ml}$) was added and cells were incubated at 37°C for 10 minutes. After that the labelled medium was removed and stored in a 50 ml Falcon tube at 37°C . 2 ml of pre-warmed PBS was added to the dishes and they were then UV-irradiated with a germicidal UVC lamp (emitting predominantly 254-nm) at the desired rate.

After UV-irradiation the [^3H] TdR medium was put back into the dishes and cells were allowed to incubate in it for a further 30 minutes and subsequently changed into normal unlabelled medium containing the four deoxyribonucleosides to a final concentration of 5×10^{-6} M. Samples were processed for DNA isolation either immediately (0 hours) or at various times after UV irradiation as described by Ryan *et al.* (Ryan *et al.*, 1991). Briefly, the cells were detached by incubating 1 minute at 37°C with 1 ml of Viokase. The cell suspension was then collected in 10 mls of medium and spun down for 5 minutes at 1300 rpm.

The cell pellet was then resuspended in $400 \mu\text{l}$ of 0.8% LMPA and the agarose+cell suspension was then sucked into a fine plastic tube (10-15 cm long \times 3mm wide) with a syringe. The tubing was then put in ice for a few minutes until agarose had solidified. Each agarose worm was then pushed out of the tubing and cut into plugs (3-5 mm long). The plugs containing the cells were then put into vials and pre-equilibrated in 2.5 ml of 0.5 M EDTA/1% Sarkosyl for a minimum of 2 hours. After 2 hours the plugs were incubated overnight at 50°C in the same buffer plus proteinase K ($50 \mu\text{g/ml}$). This allows gentle cell lysis without damaging their DNA.

Plugs were washed the next day in 10 ml 50 mM EDTA/100 mM Tris and left to wash in the cold room five times over a period of three days to remove the lysis solution and proteinase K. This method yields plugs containing radiolabelled DNA equivalent to 1×10^5 cells.

After the plugs had been washed they were separated into 3 different vials. The first

vial contained approx. 1/2 the plugs. These were used as controls. The second vial contained approx. 1/4 of the plugs and was γ -irradiated in 0.5 M EDTA/ 1% Sarkosyl (irradiations were performed with a Mainance 500C machine using a [^{137}Cs] gamma ray source at a dose rate of 1 Gy/min) whilst the third vial containing the rest of the plugs was treated with DenV.

The DenV treatment was performed as follows:

Crude extracts of T4 endonuclease V (DenV) were prepared in R. Johnson's laboratory in Cambridge using the following method. The DenV was purified from an overproducing *E.coli* strain AB2480 transfected with the plasmid ptac-DenV. An overnight culture of the bacteria was diluted 100 times and grown at 37°C in fresh L broth with ampicillin to mid-log phase ($\text{OD}_{650\text{nm}} = 0.4\text{--}0.6$). IPTG was then added to a final concentration of 0.75 mM, and the culture incubated for a further 4 hours at 30°C. Cells were then collected by centrifugation at 4,000 rpm at 4°C, sonicated in cold lysis buffer (10 mM Tris-HCl pH 7.5, 1 mM glutathione reduced, 10% ethylene glycol, 2 mM EDTA pH 8.0). Cellular debris was removed by centrifugation at 40,000 rpm for 60 minutes at 10°C, and crude extract was partially purified by phase separation in 20% w/w dextran T500, 30% w/w polyethylene glycol 600 (PEG), and about 40% w/v NaCl. The upper phase was carefully removed and dialysed using BRL dialysis membrane (MW cutoff=12–14kDa) for 6 hours at 4°C (10 mM potassium phosphate pH 6.5, 10% (v/v) ethylene glycol, 10 mM β -mercaptoethanol, 2 mM EDTA pH 8.0). Debris was removed by centrifugation at 10,000 rpm for 30 minutes at 4°C. Aliquots of the supernatant were stored at -20°C for several months. The extracts were tested for DenV and non-specific activities on a superhelical UV-irradiated plasmid, and were used at a dilution of at least 1:100.

The DNA embedded in the agarose was treated or mock-treated with DenV to cleave the DNA at sites containing CPDs. Plugs were taken out of the washing solution and pre-equilibrated in 1× DenV buffer (100 mM NaCl, 50 mM EDTA pH 8.0, 10 mM Tris pH 8.0, 1 $\mu\text{g}/\text{ml}$ BSA) for 1 hour on ice and then in the presence or absence of DenV for 30 minutes to allow the enzyme to diffuse into the plugs. They were subsequently incubated at 37°C for 1 hour. The reaction was stopped by washing in storage buffer (100 mM Tris pH 7.5, 50 mM EDTA) and stored in the cold room.

Treated or mock-treated DNA was assayed for the presence of DSBs or SSBs by neutral pulse field or alkaline gel electrophoresis, respectively.

1. Asymmetric Field Inversion Gel Electrophoresis (AFIGE) Technique.

This technique is an adaptation of the gel electrophoresis technique developed by Denko *et al.* (Denko *et al.*, 1989) and Stamato and Denko (Stamato and Denko, 1990).

The agarose plugs, treated or mock-treated with DenV, were loaded into the wells and electrophoresed in 1.5% agarose gels in TBE, 2% Ethidium Bromide for 6–8 hours under the following conditions (Ryan *et al.*, 1991):

- 125 seconds at +150 V
- 15 seconds at -300 V

After a photograph was taken, the gels were then processed so that each lane that had been run was cut into two pieces. One that included the well (DNA that had not migrated into the gel) and another one that included the rest of the lane.

Each gel wedge was put into a scintillation vial, 1 ml of 0.1N HCl was added and the gel was microwaved for 2–3 minutes until the agarose was melted. Subsequently 5 ml of scintillin was added and the [^{14}C] and [^3H] counts were counted in a scintillation counter overnight and the results analysed as follows.

Under the electrophoretic conditions described above, DNA that is smaller than 1.6 Mb enters the gel, where it migrates as a discrete band independent of the range of molecular sizes. The amount of DNA that enters the gel is a measure of the DSBs formed, and is determined by calculating the fraction of radioactive counts that are released into the gel as a percentage of the total radioactivity (%FAR) in that sample.

It has been shown that for a known dose of X-rays, the amount of DNA released from the plug is significantly reduced in S-phase cells relative to cells in other phases of the cell cycle (Iliakis *et al.*, 1991). The DNA released varies linearly with the X-ray dose for both bulk and pulse-labelled nascent DNA, but the

FAR values for the replicating DNA ($[^3\text{H}]$ -labelled) are several times lower than those of the bulk DNA ($[^{14}\text{C}]$ -labelled) (Stamato and Denko, 1990), (Ryan et al., 1991). With the AFIGE technique it is not possible to estimate the number of DSBs per unit of DNA directly, but it can be estimated indirectly by comparing the %FAR of the experimental DNA with that of the DNA exposed to a known dose of X-rays. The difference in the AFIGE assay in the response of replicating and bulk DNA to a given X-ray dose can vary in different cell strains and at various times after UV irradiation. In order to compare different samples and cell types the results are expressed as Gy equivalent DSBs and not as %FAR. The Gy equivalent of DSBs in the mock treated samples was subtracted from the values of the treated cells (for more detail see Chapter 4).

2. Alkaline Gels

The removal of cyclobutane dimers from the DNA replicating at the time of UV irradiation ($[^3\text{H}]$ -labelled) and from the rest of the genome ($[^{14}\text{C}]$ -labelled) was determined by measuring the SSB frequencies using the alkaline gel electrophoresis method. The method described here is an adaptation of the one developed by Freeman *et al.* (Freeman et al., 1986).

The number of SSBs generated by DenV represent the number of incisions at CPD sites. DenV-treated or mock-treated DNA was denatured in alkaline loading buffer (300 mM NaOH, 6 mM EDTA, 18% Ficoll, 0.15% Bromocresol Green, 0.25% Xylene Cyanol FF) for 1 hour and 20 minutes at 37°C.

0.4% agarose denaturing gels were first made in neutral buffer (0.05 M NaCl, 0.04 M EDTA) and allowed to soak in denaturing buffer (0.001 M EDTA, 0.02 M NaOH) for 30 minutes.

The gel was then run at 25 V/hour, 400 mAmps, 100 Watts overnight in denaturing electrophoresis buffer (30 mM NaOH, 2 mM EDTA). Before visualising and processing for the scintillation counter the gel was neutralised for 1 hour (250 mM Tris pH 7.5, 750 mM NaCl), stained in Ethidium Bromide (0.005% solution) for 1 hour and photographed.

Each agarose track was then cut as follows starting from the well:

- $8 \times 0.5\text{cm}$ slices
- $1 \times 1.5\text{cm}$ slices
- $3 \times 2.0\text{cm}$ slices

As markers the following samples were used.

- 50 kb lambda concatemers
- 1Kb ladder (0.5-12 kb fragments)
- 170 kb T4 DNA
- lambda digested with XhoI to give 15 and 33 Kb fragments

A marker size versus distance of migration curve is constructed using these markers and the molecular weight of the DNA contained in each of the agarose wedges was calculated and each gel wedge was then processed for the scintillation counter as described before for the AFIGE gels.

Inhibition of DNA replication by UV is determined from the sum of radiolabelled counts measured in each alkaline gel track. The sum of [^3H]-thymidine radiolabelled counts, was normalised against the sum of [^{14}C]-thymidine counts incorporated to the uniformly labelled bulk DNA. The total [^{14}C] radioactivity recovered from each gel track is used as a measure of the number of cells loaded per track.

[^3H]:[^{14}C] ratio is taken as a measure of the amount of DNA synthesis in the cells during the radioactive pulse.

The effect of UV irradiation on the molecular sizes of the daughter and parental DNA strands is estimated from the migration of the [^3H] and [^{14}C] radiolabelled DNA in alkaline gels. The average size of the parental DNA varies between different gel electrophoresis runs of the same material but the ratio between the average molecular size of the [^3H]-labelled and the [^{14}C]-labelled molecules remains constant. Therefore the changes in the average molecular size of the daughter strand after UV are expressed as changes in the ratio and not in the absolute measured molecular size values.

The frequency of endonuclease sensitive sites (ESS) in replicating and bulk genomic DNA can also be calculated from the alkaline gels.

The number of incisions introduced in the UV-irradiated DNA by the DenV at a CPD is determined from the number of single strand breaks, which is in turn determined from the average molecular size number (M_n). M_n is calculated from the radioactive profile of migration of the DNA on alkaline agarose gels. Using the two radiolabelled precursors, the relative amount, and hence the value of M_n , is determined for both the replicating and bulk DNA in each wedge. The method of calculation is a variation of the one used by Walker *et al.* (Walker, 1981) for calculating the average number of ESS from the alkaline sucrose sedimentation profiles. The distance of migration of the DNA molecules is dependent on size, and the relationship is not linear. As described above, for each gel a standard curve of migration of the size markers was calculated and the DNA size of the fragments in each gel wedge was determined.

Rough Biochemical Assays: Replication Arrest Kinetics

Cells were plated out at the appropriate density and were allowed to settle overnight in an incubator at 37°C. The following day they were put in isoleucine-deprived medium for at least 10 hours in order to arrest them in G1 phase of the cell cycle. The cells were released from the G1 block 6 hours before irradiation by putting them back into normal growth medium. After 6 hours the cultures, which were well into S phase, were mock-irradiated or irradiated with a hand-held UV lamp with the desired UV fluence. The cells were then processed immediately or put into normal medium and processed at different times after UV.

Typically, cells were rinsed in serum-deprived medium and pulsed 15 minutes in [^3H]thymidine-containing (2 $\mu\text{l}/\text{ml}$) serum-deprived medium at 37°C. After 15 minutes, the cells were rinsed in sterile PBS, lysed in a 1%SDS solution, and TCA precipitated with 1/2 volume of 20% (v/v) TCA pre-chilled at 4°C. Samples were kept at 4°C until needed.

The samples were vacuum-filtered by passing them through 2.4 cm GF/C microfibre filters (Whatman). Each filter was put into a plastic scintillation vial and 2 mls of scintillation liquid was added to it. They were put into the scintillation counter and the [^3H] incorporation was measured as DPM counts.

2.2.13 Cytology: Morphometric Analysis

Semiautomated System for Hepatocyte Nuclear Area Measurement

Liver sections from ERCC1+/+ and ERCC1+/- aged animals were collected, fixed and embedded in paraffin. Sections (3 μ m) were cut and these were stained with haematoxylin and eosin. In order to measure the size of each one of the hepatocyte nuclei present in a field, IBAS20 (Kontron Elektronik GMBH, Munich; IBAS software v.2.0) was used. This system is a semiautomatic image analysis package available at the Imaging Unit in the Pathology department of Edinburgh University. Briefly, IBAS20 digitalises the image you desire (i.e., random field of a liver section), processes it and discriminates between the different structures on the section (i.e., hepatocyte nuclei, white blood cell nuclei, background connective tissue) through colour intensity differences in them. The computer produces a graphical representation of the measurements per slide (approx. 15 fields) and works out the statistical parameters for the different values of the area, the minimum diameter (D1) and the maximum diameter (D2) of the nuclei. All images were acquired using a 100 \times /NA 1.4 oil immersion objective lens.

This system has the advantage of standardising any error that may occur as opposed to errors made during manual measurements which are non-reproducible such as bad visibility of nuclei due to imperfect staining, loss of accuracy in measurements, etc., and has the additional convenience of being much faster and giving out automatic statistical analysis of each slide separately. The results were analysed with the Sigma Plot package.

AxioHOME Morphometric System

This system was used to perform the study of Ki67, BrdU and area size distribution in young animals (up to 21 days of age). Contrary to the semiautomated system used before, the AxioHOME system (Zeiss) is not automatic and each measurement of the perimeter of the hepatocytes has to be done manually (Brugal, 1992). Basically, the software used, Basic Morphometry, allows the 'drawing' around the periphery of each nucleus in a field and the length of the perimeter is entered in the computer, which in

turn works out the area in μm^2 's for each one of the measurements.

Typically, 100 \times /NA 1.4 oil immersion objective lens was used for the measurements. 10 fields, containing approx. 50–70 nuclei each, were measured for the Ki67 and BrdU–stained sections. For the area measurements and binucleate hepatocyte numbers (haematoxylin/eosin sections) all the nuclei in a field were measured and 5 fields were analysed per animal. The results were analysed with the Sigma Plot package.

Chapter 3

Liver Morphology and Immunocytology Studies

3.1 Introduction

p53 has been extensively studied in relation to its effect on cell cycle regulation. On the detection of DNA damage, p53 levels are elevated through an increase in the p53 protein half-life. p53 is capable of inducing the transcription of downstream genes, such as p21 (Bates and Vousden, 1996), and this p53-mediated response to genotoxic stress is characterised by the arrest of cell cycle progression at both G1 and G2 checkpoints, and in some cell types it can also lead to apoptotic cell death.

p53 and p21 have also been implicated in cellular ageing (Harvey et al., 1993; Noda et al., 1994; Atadja et al., 1995; Bond et al., 1994; Bond et al., 1995). p53 levels accumulate as diploid fibroblasts age, with a corresponding increase in p21 levels, which in turn correlate with a slowing of cellular growth.

The morphology of liver tissue in ERCC1-deficient mice is already well characterised (McWhir et al., 1993a). Their liver presents characteristics that indicate premature ageing (i.e., elevated numbers of polyploid cells) as well as increased levels of p53 in hepatocytes. However, it is not clear what the role of p53 is, if any, in the process leading to the observed polyploidy and aneuploidy in the ERCC1-deficient liver.

If the increase in the levels of p53 is responsible for ploidy changes in ERCC1-deficient liver, the possibility exists that the ERCC1-deficient liver phenotype might be rescued

in mice deficient in both ERCC1 and p53 (double null mice).

In order to study this, a number of experiments were carried out using 3 week-old mice of the following genotypes: p53 wt/ERCC1 wt (wt/wt), p53 null/ERCC1 wt (p53hom/ERCC1wt), p53 wt/ERCC1 null (p53wt/ERCC1hom), p53 null/ERCC1 null (p53hom/ERCC1hom). The choice of age of the mice for this study is due to the severe phenotype of the ERCC1-deficient animals, which do not survive longer than 3 weeks (McWhir et al., 1993a). As will be discussed later, this is also the case for the double null animals.

Ki67 (as a marker for cell proliferation), BrdU (as a marker for DNA synthesis) and morphological studies (both nuclear area measurements and binucleate cells count) in haematoxylin/eosin (h/e) liver sections were used to gain a better understanding of the cell cycle status of the hepatocytes.

The morphology studies were mainly focused on the measurement of the nuclear area of the hepatocytes, since this feature is directly related to ploidy (Epstein, 1967a). The area of the hepatocyte nucleus becomes bigger as the nucleus acquires increasing degrees of ploidy, which in turn is related to the age of the animal (the older the mouse the higher the percentage of cells with larger/polyploid nuclei). The measurement of nuclear area for the determination of ploidy in hepatocytes and its relationship with the ageing process in several species has been studied extensively (Epstein, 1967a; Epstein, 1967b; Brasch, 1980; Medvedev, 1986; Bohm and Noltemeyer, 1981; Kudryavtsev et al., 1993).

Following this line of thought, it was of interest to see if there was any correlation between the ERCC1 status and the ageing process in mice. Clearly, the fact that ERCC1-deficient animals died before weaning did not allow a study of the effect of a total ERCC1 deficiency at later stages in the mouse development. On the other hand, it was known that ERCC1+/- mice had a normal appearance and survived as long as their wild type counterparts. It was thus possible to address a related question by studying the effect of a partial deficiency of ERCC1 in the livers of aged animals. Is the effect of an ERCC1 deficiency cumulative? Is it therefore possible that aged ERCC1+/- animals show the same liver phenotype as the young ERCC1-/- ones?

In order to answer these questions a number of both ERCC1+/+ and ERCC1+/- animals were collected at an advanced age (ranging from 15 to 31 months) and their liver morphology, i.e. the area of the hepatocyte nuclei, was compared.

3.2 Results

3.2.1 Aged ERCC1 heterozygote mice do not show any sign of aberrant liver morphology with increasing age

Liver sections from ERCC1+/+ and ERCC1+/- aged animals were collected, fixed and embedded in paraffin. Sections (3 μ m) were cut and these were stained with haematoxylin and eosin before analysing them using the Semiautomated IBAS20 microscope system. Measurements of the area of each hepatocyte nucleus detected by this system were taken and the results were analysed using the Sigma Plot package. For more details see Materials and Methods, section 2.2.13. The results of the study are summarised in Fig. 3.1.

Enlargement of hepatocyte nuclei as a consequence of ageing is a common aspect of the liver physiology. As the liver ages the hepatocytes tend to the polyploidisation of their nuclei. It has been proposed that this is a defense mechanism against intrinsic DNA damage occurring in the liver (see Discussion). Since the liver is an organ with low levels of cell proliferation, hepatocytes would instead replicate their DNA as a way of ensuring the sustainability of essential genes. This process would acquire a bigger significance with increasing age as the endogenous DNA damage would accumulate with time.

As can be seen in the graph presented below there is no significant difference between the distribution of hepatocyte nuclear sizes in aged ERCC1+/+ and ERCC1+/- mice. These data suggest that the absence of only one copy of ERCC1 does not affect the liver morphology with time. A possible explanation for this is that the requirement for ERCC1 may be very small in the cells and a reduction in the production of ERCC1 protein does not affect the function of NER in the hepatocytes. Only when ERCC1 is totally absent is NER disrupted and the aberrant morphology appears.

Although this type of data analysis is different from the one used for the experiments

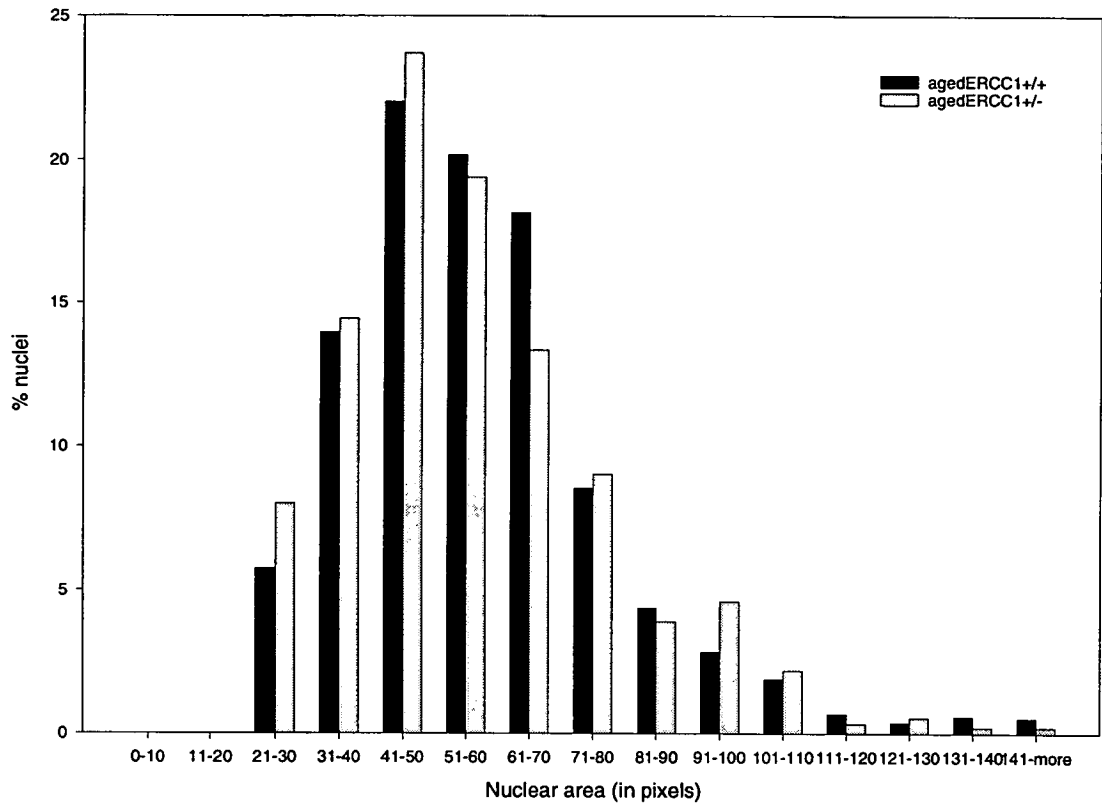


Figure 3.1: Distribution of the size in pixels of hepatocyte nuclei belonging to ERCC1+/+ (animals sampled=23, 9 male+14 female) and ERCC1+/- (animals sampled=34, 18 male+16 female) aged animals. Both genotypes presented an equivalent age distribution: ERCC1+/+ animals aged from 15-24 months=68.2%, ERCC1+/- animals aged from 15-24 months=71.9%; ERCC1+/+ animals aged from 24-31 months=31.8%, ERCC1+/- animals aged from 24-31 months=28.1%.

described below, the general appearance of the profiles of nuclear area distribution can be compared. In fact, the profile displayed by the distribution of nuclear areas in both aged ERCC1+/- and ERCC1 wild type mice is similar to that displayed by the young ERCC1-/- deficient animals (see Fig. 3.3). All three present a high number of different size classes indicating a loss of the nuclear area uniformity, which is a characteristic of young wild type livers (see Fig. 3.3). What is more, both profiles belonging to the two aged categories and the ERCC1-deficient profile show a clear shift to the bigger area classes compared to the young wild type liver profile, indicative of an increase in polyploid hepatocyte nuclei (see sections below and Discussion).

3.2.2 The absence of p53 does not rescue the ERCC1-deficient phenotype

It was clear from early observations of the survival of the different classes of mice that animals that were both ERCC1-deficient and p53-deficient did not survive longer than their ERCC1-deficient counterparts. This was the first clear indication that the absence of p53 did not rescue the overall phenotype associated with ERCC1 deficiency. Double null mice were also runted and died before weaning. It is important to note that the frequency at which this particular genotypic class was detected was extremely low. In the course of experiments that have lasted 4 years, where several hundred animals from crosses between double heterozygotes have been genotyped by tail cutting at 3 weeks, double nulls have been detected at far less than the expected ratio of 1:16. Some of these have then died before liver samples could be collected. This has been the main reason for the low numbers of double null samples used in these experiments.

In order to see if the liver phenotype was rescued, preliminary studies of h/e liver sections from these mice revealed that in fact, the appearance of the ERCC1 null and the double null livers was very similar (see Fig. 3.2 B and D). Indeed, the nuclear uniformity had been lost in p53wt/ERCC1hom and p53hom/ERCC1hom livers, with both showing enlarged nuclei in addition to the smaller size characteristic of wild type. This fact pointed towards a failure of p53 absence to rescue the ERCC1-deficient liver phenotype.

Binucleation was another morphological aspect that was investigated in these livers. Binucleation is a feature that has been related to normal liver development and ageing along with polyploidisation. In normal mouse liver development, the number of binucleate and polyploid cells increase with increasing age, concomitant with a decrease of mononucleate, diploid cells (Epstein, 1967a). Despite overall similarities in the appearance of the ERCC1 null and the double null livers there was a suggestion from initial observations that the number of binucleate cells was elevated in the double null livers compared to the ERCC1-deficient livers (see Fig. 3.2 B and D).

Given the biological significance of binucleation and polyploidisation in the development of mouse liver, it was decided to carry out a more systematic study of these

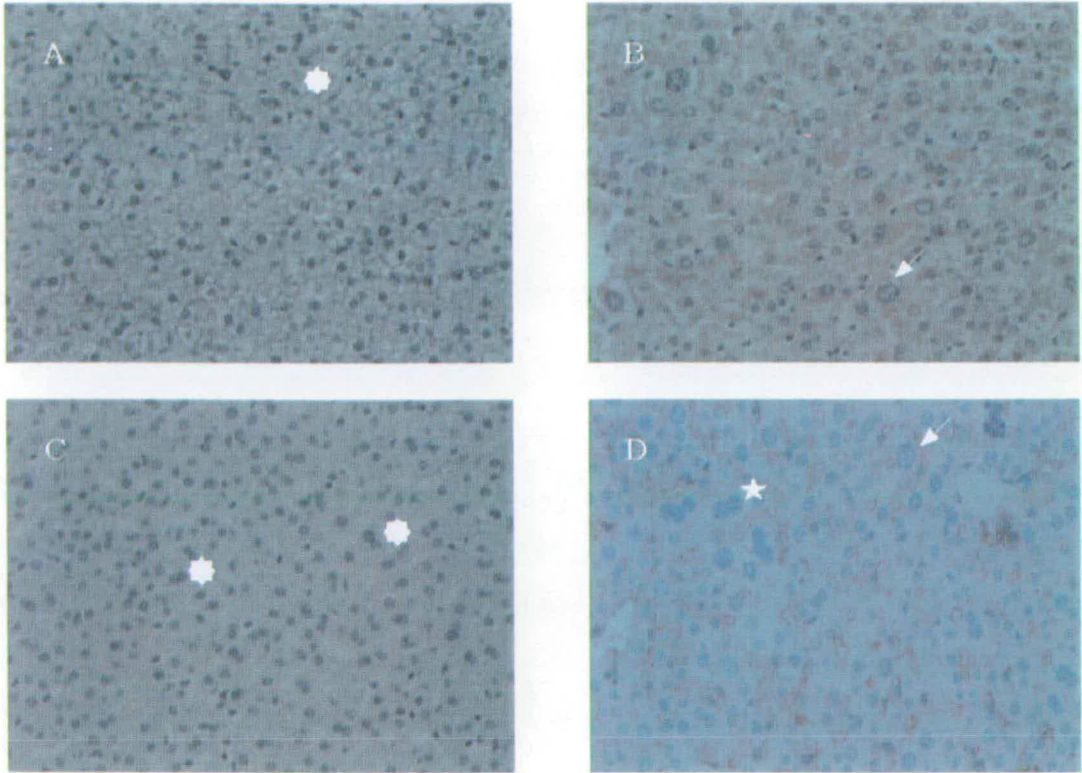


Figure 3.2: Morphology of the four different genotypic classes of liver from age matched mice (3 weeks old). Photographs of liver sections were taken from haematoxylin stained sections under the microscope using a 40 \times magnification lens. A) wild type liver showing the typical uniform distribution of small nuclei seen in young animals. Note its similarity to the distribution seen in panel C. Eight-point star is situated next to a normal size binucleate cell. B) p53wt/ERCC1hom liver. This picture shows a wide distribution in nuclear areas. Arrow points towards a polyploid (giant) nucleus. C) p53hom/ERCC1wt liver showing a young wild type-like nuclear distribution. Eight-point stars are situated next to normal size binucleate cells. D) p53hom/ERCC1hom liver. Note the abnormal distribution of nuclear sizes and its similarity to panel B. Arrow point towards a polyploid (giant) nucleus and star is situated next to a polyploid binucleate cell.

aspects and in order to do so, the size of the hepatocyte nuclei and the number of binucleate cells were determined for the haematoxylin/eosin sections of liver belonging to the four genotypes: p53wt/ERCC1wt (wt), p53hom/ERCC1wt, p53wt/ERCC1hom and p53hom/ERCC1hom. Only cells that appeared as unequivocally parenchymal in origin were counted, taking care to avoid the areas surrounding the portal veins, where there is an increased number of non-parenchymal cells. This section deals with the results obtained from the study of the hepatocyte nuclear area distribution in the aforementioned livers and the next section deals with the incidence of binucleation in the same livers.

The distribution of the nuclear area sizes is shown in Fig. 3.3. The wild type profile (top panel) shows a distribution of areas characterised by a mode of 20-30 μm^2 and a maximum area of 70 μm^2 . The number of nuclei that fall within the last two nuclear categories (from 50-70 μm^2) is so low (1% of the total nuclei number) that 50 μm^2 is the cut-off area that was chosen in order to determine abnormal or big (polyploid) nuclei. This distribution coincides with the one that has already been reported by McWhir et al.(1993) and Weeda et al.(1997).

Out of the four genotypes, only the p53hom/ERCC1wt livers present the same distribution as the wild type ones (see figs.3.3 and 3.2). This suggests that p53 absence does not affect liver morphology and this is confirmed again by the observations made in the double null livers.

In both the ERCC1-deficient livers and the double null livers the size distribution profiles present a more spread-out shape, which indicates a shift of these hepatocytes towards the bigger nuclear areas. As similar pattern of nuclear area distribution in ERCC1 null mice is reported by Weeda et al.(1997). In fact, we observe that the mode in these two categories has increased from 20-30 to 30-40 μm^2 , and the biggest nuclei reach up to 200 μm^2 . Also, the percentage of nuclei which are bigger than 50 μm^2 has increased for both the ERCC1-null and the double null genotypes (33.7% and 34.4%, respectively). This percentage remains low for the p53 null livers (5.36%). Overall, there is no clear difference in the distribution of nuclear areas in ERCC1 -deficient and the double null livers. What is more, both of them resemble the profiles expected for an aged liver (see Fig.3.1). The implications of this observation are discussed below

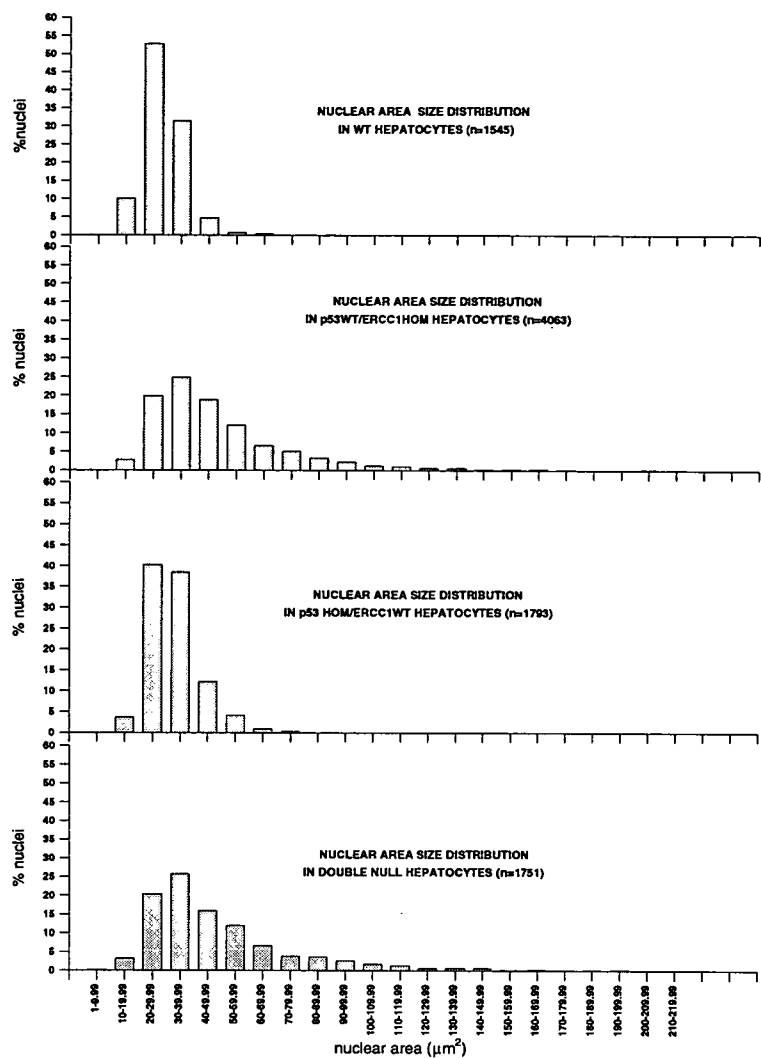


Figure 3.3: Nuclear area size distribution in the four different genotypic classes of hepatocytes from 3 week-old mice. The areas were calculated as described in the Materials and Methods, section 2.2.13. The total number of nuclei counted per genotype is represented by n.

(see Discussion and sections below).

The results obtained here indicate that the absence of p53 does not rescue the ERCC1-deficient phenotype. Therefore, the aberrant ploidy patterns seen in these hepatocytes are most likely created through a p53-independent mechanism.

3.2.3 Binucleation is inhibited in ERCC1-deficient hepatocytes

As mentioned before, there have been previous studies of distribution of ploidy classes of hepatocytes in mouse livers (Swiss mouse) detailing the changes in ploidy with increasing age (Epstein, 1967a). One of the characteristics of such changes is the pattern of mononucleate and binucleate cells co-existing at a given time. The older the mouse the higher the percentage of high ploidy (4n, 8n) binucleate cells present and the lower the number of diploid (normal) cells.

In order to study this aspect of liver cytology, binucleate cells were counted and divided into two categories: normal (area < 50 μm^2) and big/polyploid (area > 50 μm^2) (Fig.3.4). The basis behind this rationale has already been discussed in the previous section.

From this graph it is clear that there is a decreased number of binucleate cells in the ERCC1 null livers compared to the other genotypes (see also Figs. 3.2 A, B, C and D). Both the wild type and the p53^{-/-} ERCC1^{+/+} livers have similar percentages of binucleate hepatocytes (8% and 10% respectively). In these two genotypes there is no evidence of big (high ploidy) binucleate cells. In ERCC1^{-/-} livers the percentage of total binucleate cells is the lowest of the four types (3%), with 44% of that number being comprised of high ploidy nuclei. In the case of the double null livers, the total number of binucleate cells is increased, being similar to that of the wild type or the p53 deficient tissues (7%), and a high percentage (61%) of those binucleate cells are polyploid.

Thus, binucleation seems to be impaired in the absence of ERCC1 and this block is rescued by the absence of p53. The mechanism responsible for this ERCC1 effect remains to be determined. It is noteworthy however, that the levels of p53 are only elevated in the ERCC1^{-/-} livers and that this seems to correlate with a decrease in binucleation. Since binucleation happens through repeated replication rounds in the absence of cell division and is the previous step to polyploidisation in the development and differentiation of the liver, this characteristic is of relevance to the liver phenotype observed in ERCC1-deficient mice and a possible link between ERCC1, replication control and liver development.

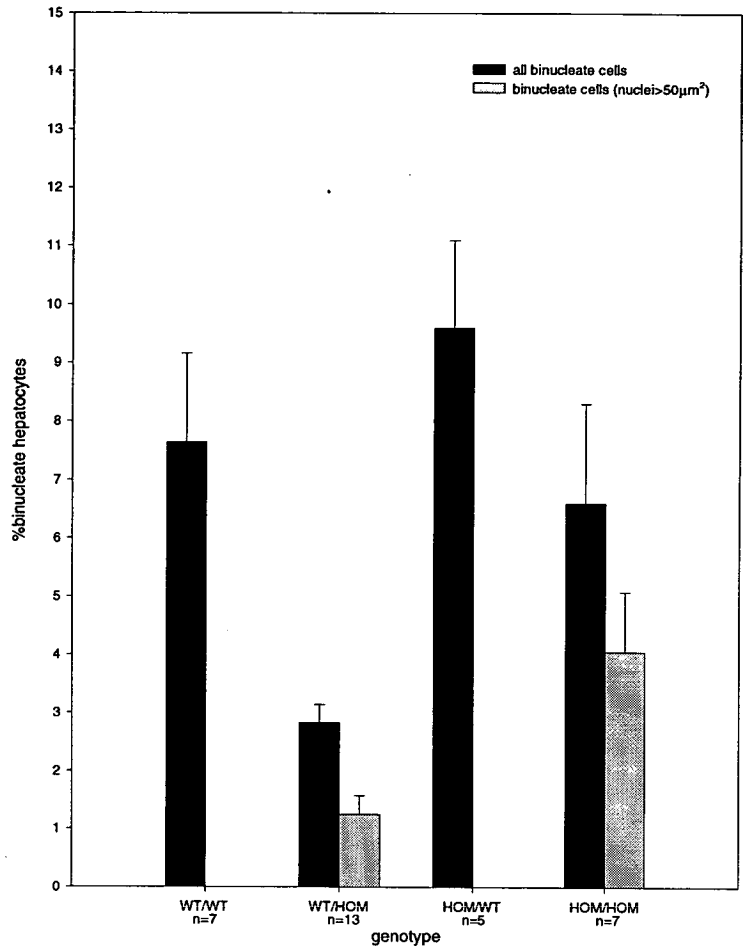


Figure 3.4: Percentage of binucleate cells in the four different genotypic classes of liver from 3 week-old mice. Big nuclei (when present) are represented by the second (grey) bar in each genotype. wt/wt stands for p53wt/ERCC1wt; wt/hom stands for p53wt/ERCC1hom; hom/wt stands for p53hom/ERCC1wt and hom/hom stands for p53hom/ERCC1hom. Standard errors are shown. n indicates the number of animals sampled per category.

3.2.4 The absence of p53 rescues the replication block in ERCC1 null livers

Sections of liver were also stained with an anti-BrdU antibody in order to establish the replication index for each one of the genotypes.

The results obtained for this study are summarised in the following graph (Fig. 3.5). Because ERCC1-deficient and double null animals are runted, it was decided to use

rare runt wild type and p53 nulls as controls for this experiment. For comparison, BrdU incorporation was also determined for normal (non-runt) wild type animals.

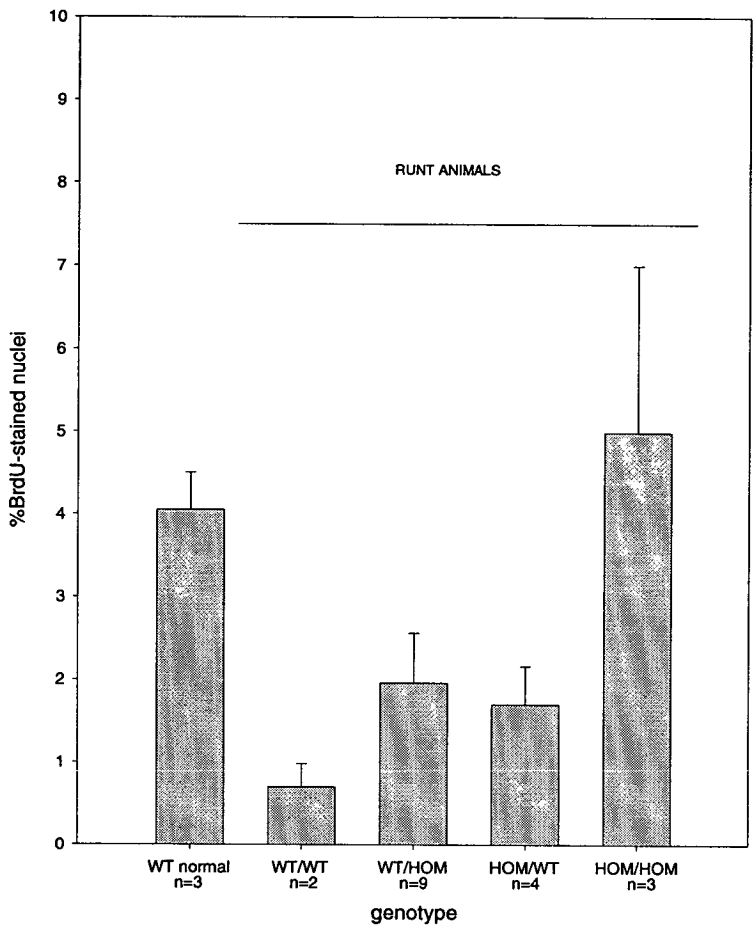


Figure 3.5: Percentage of BrdU-stained cells in the four different genotypic classes of liver from 3 week-old mice. Standard errors are shown. wt/wt stands for p53wt/ERCC1wt; wt/hom stands for p53wt/ERCC1hom; hom/wt stands for p53hom/ERCC1wt and hom/hom stands for p53hom/ERCC1hom. n indicates the number of animals sampled per category.

Overall, the labelling index for the runt wt/wt, p53hom/ERCC1wt and p53wt/ERCC1 hom livers is very low (less than 2%) (see Fig. 3.6). This can be explained by the fact that all animals participating in this study were runt and therefore suffer from an impairment of growth. As expected, the level of replication in normal-sized wild type animals was higher (4%).

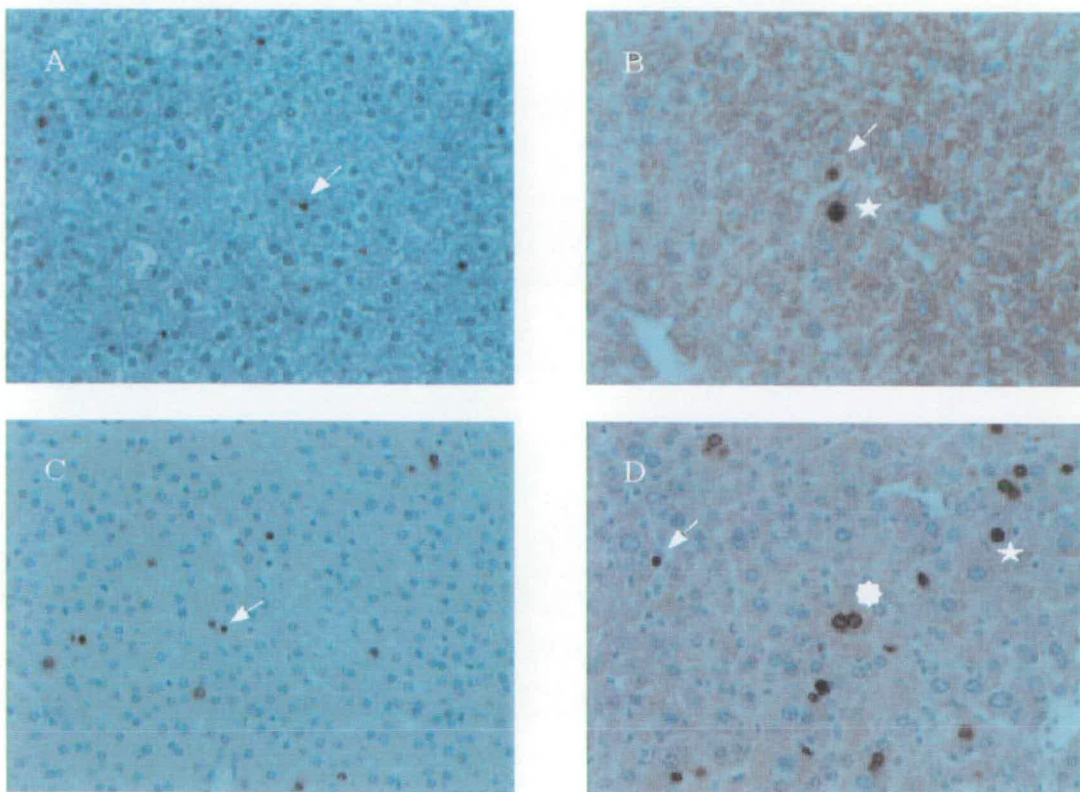


Figure 3.6: Typical BrdU staining patterns for the four different genotypic classes of liver from age matched mice (3 weeks old). Photographs of liver sections were taken from BrdU-stained/haematoxylin counterstained sections under the microscope using a 40 \times magnification lens. A) wild type liver. Arrow points towards a normal size BrdU-stained nucleus. B) p53wt/ERCC1hom liver. Arrow points towards a normal size BrdU-stained nucleus. Star is situated next to a BrdU-stained polyploid (giant) nucleus. C) p53hom/ERCC1wt liver. Arrow points towards a normal size BrdU-stained nucleus. D) p53hom/ERCC1hom liver. Arrow points towards a normal size BrdU-stained nucleus. Star is situated next to a BrdU-stained polyploid (giant) nucleus and eight-point star is situated next to a BrdU-stained polyploid binucleate cell.

Although replication levels in ERCC1-deficient livers are less than in non-runted wild type animals, when compared to runted wild type animals there is no significant detrimental effect in terms of replication. In runted p53^{-/-} livers the levels of replication are not rescued to normal wild type (non-runted) levels. It is possible that in these rare p53 null runted livers, the control of replication is carried out in the absence of p53. This suggests that under circumstances where there is efficient DNA-repair (no genotoxic stress), p53-independent pathways control the replication process.

However, in the p53^{hom}/ERCC1^{hom} tissues the level of replication has doubled compared to all other runted genotypic classes, reaching normal levels. This seems to indicate that double null hepatocytes proceed with the replication of their DNA in the absence of a proficient DNA repair mechanism.

Once the difference in the replication index of the different liver classes was established, it was of interest to determine which subpopulation of hepatocytes, i.e., diploid, or polyploid, were responsible for the increase of BrdU incorporation in double null livers. In order to investigate this, studies to determine the size distribution of BrdU-positive nuclei were carried out (a summary graph is shown in Fig. 3.7).

When the graphs corresponding to the BrdU-positive nuclei from the different genotypic classes are compared (Fig. 3.7) it can be seen that in the double null (p53^{hom}/ERCC1^{hom}) livers the distribution of sizes of BrdU positive nuclei is shifted towards the bigger nuclei when compared with the other genotypes (see also Fig. 3.6 D). What is more, if the size distribution of BrdU-stained nuclei is compared to the general nuclear size distribution in the four different genotypic classes (Fig. 3.7) it is clear that although the distributions of BrdU-stained nuclei in both the wild type, the p53 null and the ERCC1 null livers correspond with the general area distribution for the same genotypic classes, this is not the case for the double null livers.

In this latter case, it is apparent that the replicative potential of the liver lies within the polyploid subpopulation of hepatocytes (shown by a distribution of BrdU-stained nuclei that is shifted towards the bigger/polyploid nuclei) (see Fig. 3.7). If this is taken together with the fact that the percentage of BrdU-stained nuclei in these livers is higher than that seen in any of the other runted animals and has reached levels

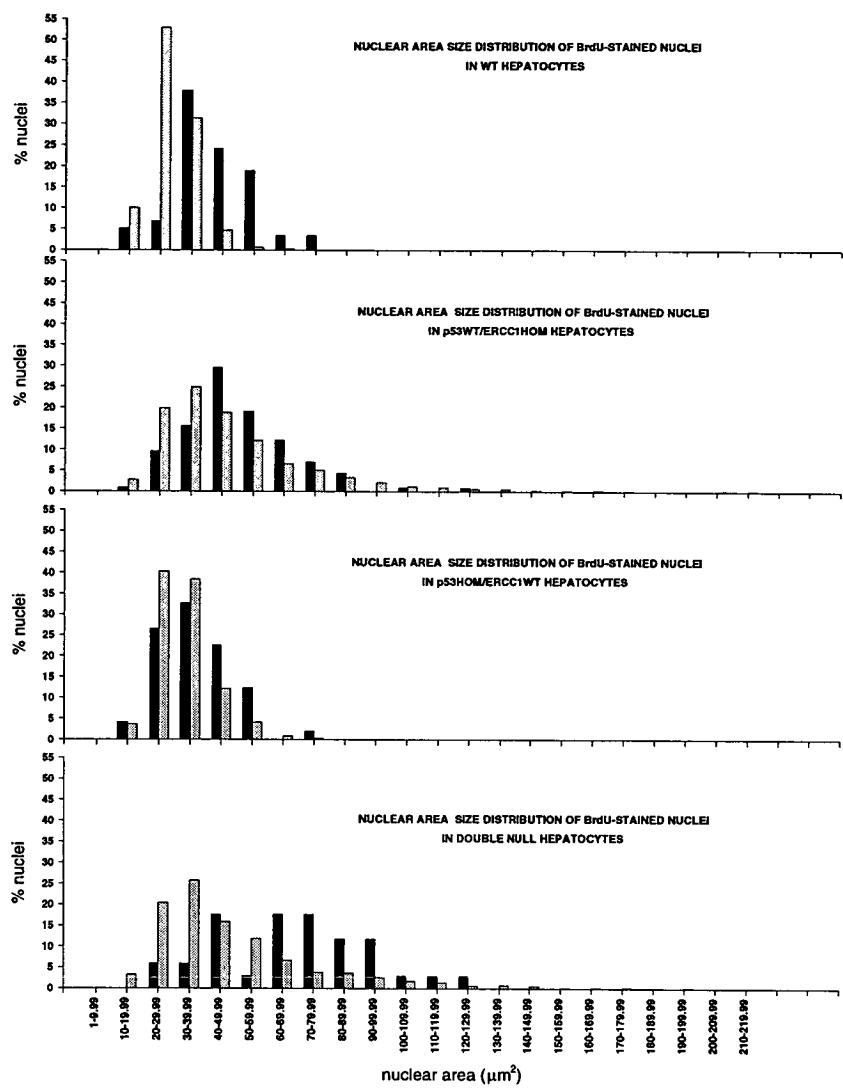


Figure 3.7: Nuclear area size distribution of BrdU-stained nuclei in the four different genotypic classes of hepatocytes from 3 week-old mice (black bars). For comparison purposes, the general nuclear area size distribution of the hepatocytes corresponding to each genotypic class is also shown (grey bars). The areas were calculated as described in Materials and Methods, section 2.2.13.

equivalent to that of normal wild type livers, it is reasonable to think that those nuclei that have ‘gained’ a replicative status are the polyploid nuclei. In addition, this ‘gain of replicative function’ effect is not seen in $p53^{-/-}$ $ERCC1^{+/+}$ cells which reinforces the idea that in this system there is a link between NER and cell cycle regulation. Indeed, NER and cell cycle control can be brought together in the following model that would explain the observations in the double null livers: Hepatocytes are subject to high levels of endogenous damage. Although double null hepatocytes lack NER,

they probably escape the arrest at the G2 checkpoint of the cell cycle (see section 3.2.6) due to their lack of p53. This allows these cells to go through additional rounds of replication, which results in the higher level of BrdU-labelling seen in these livers.

3.2.5 The ratio of (G1+G2+M) cells to S cells is elevated in ERCC1-deficient hepatocytes

Ki67 staining was used to determine the amount of cycling cells in each one of these four categories of liver tissue. Scoring was performed in the same manner as for BrdU (see Materials and Methods). The results are shown in Fig.3.8.

Although Ki67 has traditionally been reported to stain cycling cells only, it is important to note that recent studies indicate that cells that overexpress p21 or p53 remain positive for Ki67 staining even though they are arrested (vanOijen et al., 1998). Cells at G0 are not stained by Ki67.

As expected the percentage of cycling cells in the runtcd wild type mice is low (an average 2.36%), which is consistent with the runtcd phenotype and the cycling characteristics of liver cells in general. This is in contrast with the other runtcd genotypes. In the case of p53 null livers, the percentage of cycling cells has increased compared to the wild type livers. In this particular genotype, Ki67 would be expected to stain only cycling cells and therefore, this seems to be a good reflection of what would be expected of cells that do not possess functional p53, i.e., poor regulation of cell cycle progression, which ties in with the observed increased frequency of cancers seen in these mice.

In order to be able to discuss the results obtained for the rest of the genotypic classes studied it is important to take into account a number of considerations.

- In these livers, both cycling and non-cycling cells (arrested cells) are stained by Ki67.
- Cells in all stages of the cycle are stained.

Since the results of the BrdU staining procedure allow us to know exactly how many of the cycling cells are in S, it is possible to derive the percentage of cells that are either

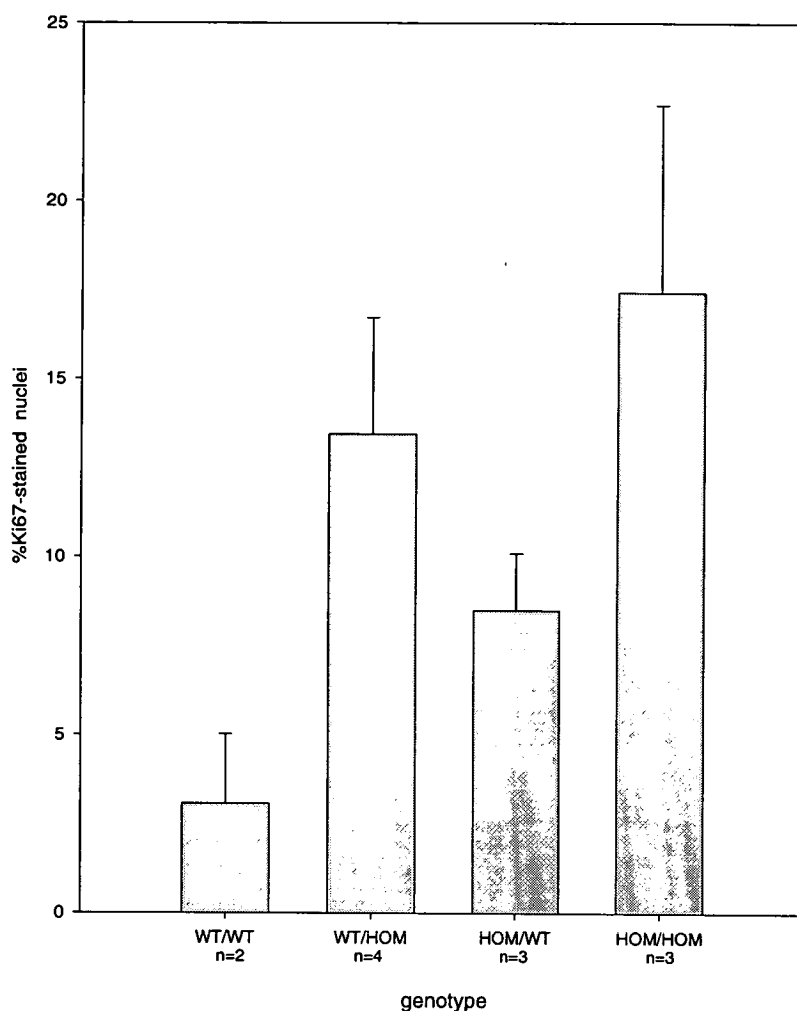


Figure 3.8: Percentage of Ki67-stained cells in runted animals of the four different genotypic classes of liver from 3 week-old mice. Standard errors are shown. wt/wt stands for p53wt/ERCC1wt; wt/hom stands for p53wt/ERCC1hom; hom/wt stands for p53hom/ERCC1wt and hom/hom stands for p53hom/ERCC1hom. n indicates the number of animals sampled per category.

in G1, G2 or M using both sets of data. These percentages are calculated in Table 3.1 and a graph showing the combined results from the BrdU and the Ki67 staining procedure is also shown below (see Fig. 3.9).

In this analysis only the population of cells within the cell cycle, i.e., those cells that are either cycling (G1,S,G2 or M cells) or cells that are arrested, is considered. The G0 population of hepatocytes is not included in the calculations. Both ERCC1 null and double null livers show a very similar increase in the percentage of cycling cells

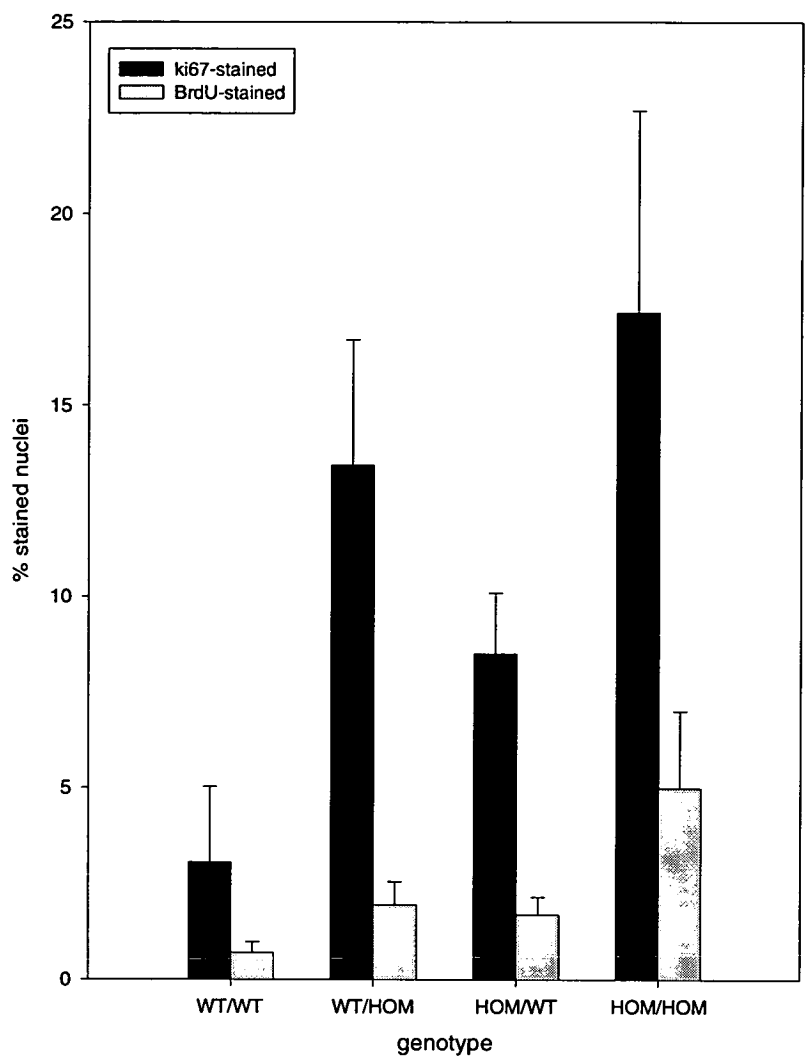


Figure 3.9: Percentages of Ki67- and BrdU-stained cells in the four different genotypic classes of livers from 3 week-old mice. Standard errors are shown. wt/wt stands for p53wt/ERCC1wt; wt/hom stands for p53wt/ERCC1hom; hom/wt stands for p53hom/ERCC1wt and hom/hom stands for p53hom/ERCC1hom.

compared to the wild type controls. However, when the ratio of G1+G2+M cells to S cells is compared, it is clear that it is much higher in the ERCC1-deficient cells and it is closer to normal, wild type levels in the double null livers. Such a difference can be explained if we think that the increase in cells other than in S phase in the ERCC1-deficient livers is due to all the hepatocytes that are arrested in G2 (see next section). In double nulls, due to the lack of p53, this arrest is not effective and the ratio of G1+G2+M cells to replicating cells is not affected. Again, this ratio is slightly elevated for p53-deficient livers, but in this case, since neither p21 nor p53 levels are

<i>Genotype</i>	<i>%BrdU</i>	<i>%Ki67</i>	<i>%(G1+G2+M)</i>	<i>%(G1+G2+M)/%S</i>
Wild type	0.7	3.06	2.36	3
p53 wt/ERCC1 hom	1.96	13.45	11.49	6
p53 hom/ERCC1 wt	1.70	8.5	6.8	4
p53 hom/ERCC1 hom	4.99	17.45	12.46	2.5

Table 3.1: Summary of the data obtained from the BrdU- and the Ki67- staining of liver sections from the four different genotypic classes of 3 week-old mice. %BrdU and %Ki67 represent the percentage of BrdU- and Ki67-positive cells; %(G1+G2+M) is the difference between the percentage of Ki67-positive cells and the percentage of BrdU-positive cells; %(G1+G2+M)/%S is the ratio of the percentage of cells in G1+G2+M to the percentage of cells in S.

elevated, this increase in the ratio of G1+G2+M cells to S cells is only due to cells cycling out of control, with no contribution from arrested cells (see discussion above).

When the distribution of the nuclear area sizes of the Ki67-positive nuclei is looked at (Figs. 3.10 and 3.11), it is noticeable that in the double null livers, those cells that are cycling show a tendency towards bigger sizes compared to the distribution shown for the general hepatocyte population. As it has already been discussed, this is also the case for the BrdU-positive nuclei in these double null livers where the effect was more pronounced. If this is taken into account, it is logical to think that the shift observed in the Ki67-positive double null cells is due to the bigger sizes of nuclei that are replicating (BrdU-positive nuclei). In the case of ERCC1-defective nuclei, both the Ki67-stained and the BrdU-stained nuclear populations show the same area size distribution as that of the general population. Since the percentage of BrdU-stained nuclei is so low in these ERCC1-deficient livers (less than 2%) the vast majority of Ki67 cells belong to the non-replicating category (%(G1+G2+M)=11.49%) (see Table 3.1; also see next section for further support for this argument), rather than S.

3.2.6 ERCC1-deficient hepatocytes are arrested at G2

In order to see what mechanisms lay behind this increase of the non-S hepatocyte population in the ERCC1-deficient livers, it was necessary to obtain direct proof of which point of the cell cycle these cells were at. This was achieved using centromeric staining of hepatocytes. Briefly, fresh liver cells were stained with a NR antibody

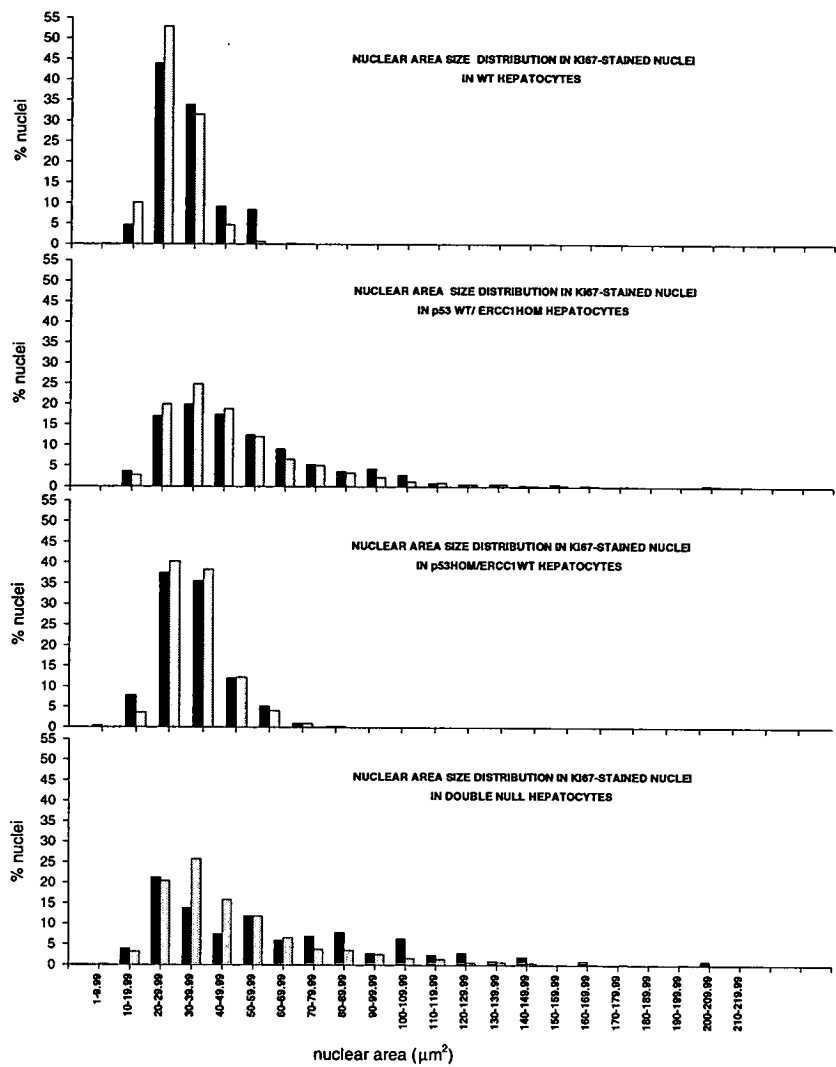


Figure 3.10: Nuclear area size distribution of Ki67–stained nuclei in the four different genotypic classes of hepatocytes from 3 week–old mice (black bars). For comparison purposes, the general nuclear area size distribution of the hepatocytes corresponding to each genotypic class is also shown (grey bars). The areas were calculated as described in Materials and Methods, section 2.2.13.

which recognises all three cenpA, cenpB and cenpC antigens, counterstained with DAPI and analysed under a deconvolution microscope (see Materials and Methods, section 2.2.11). The end result is the fluorescent staining of centromeres in the cells and the results obtained are presented in the following Figure.

Fig. 3.12(a) represents a wild type 2n hepatocyte. Each one of the red spots represents a centromeric structure. Since the majority of the centromeres appear as single structures (single red dots) it is concluded that the nucleus is at G1. This is true for

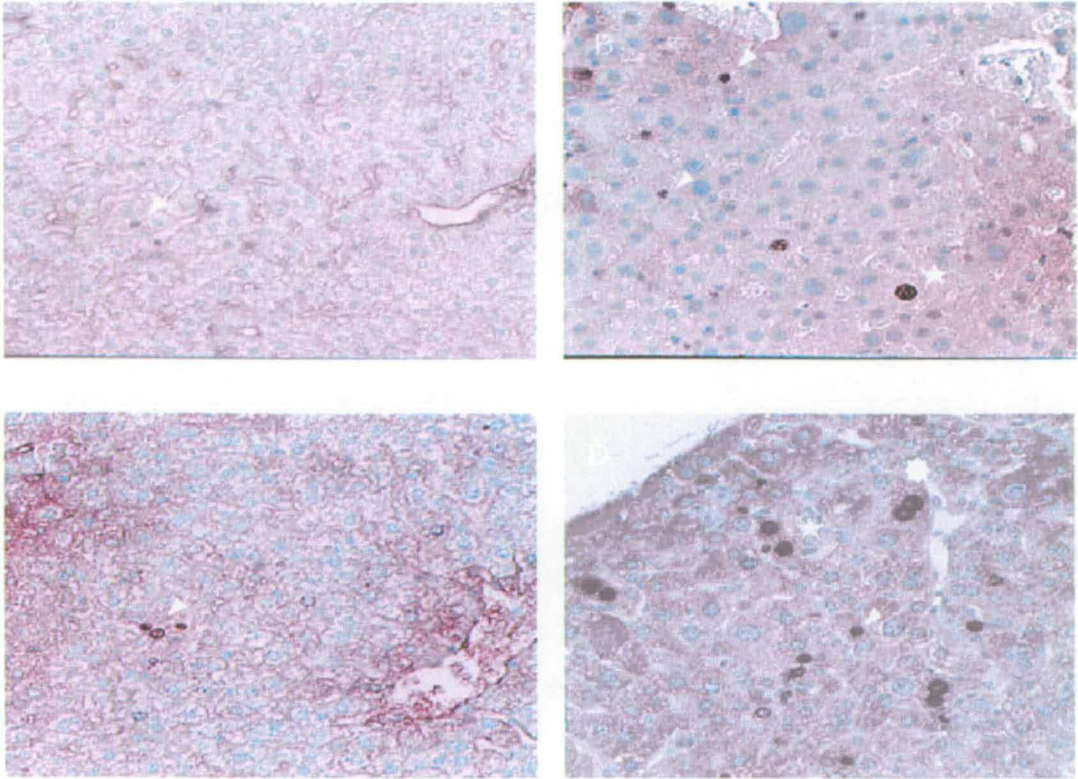
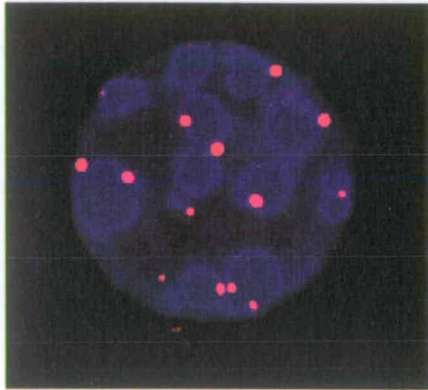
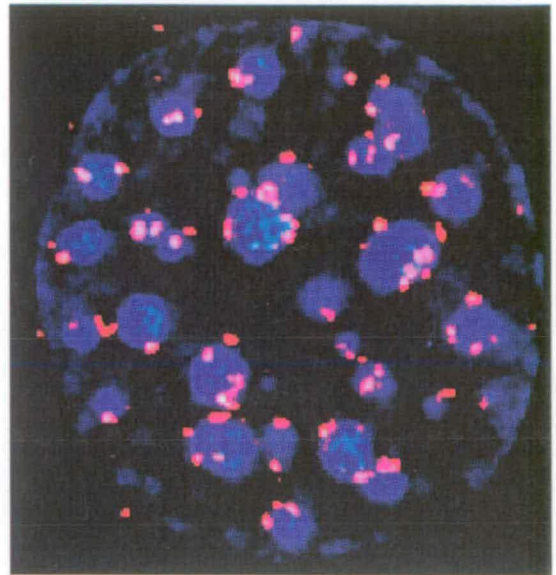


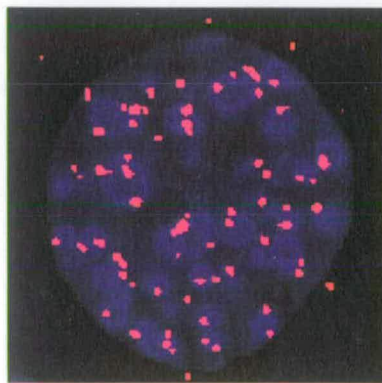
Figure 3.11: Typical Ki67 staining patterns for the four different genotypic classes of liver from age matched mice (3 weeks old). Photographs of liver sections were taken from Ki67-stained/haematoxylin counterstained sections under the microscope using a 40 \times magnification lens. A) wild type liver. Arrow points towards a normal size Ki67-stained nucleus. B) p53wt/ERCC1hom liver. Arrow points towards a normal size Ki67-stained nucleus. Star is situated next to a Ki67-stained polyploid (giant) nucleus. C) p53hom/ERCC1wt liver. Arrow points towards a cluster of normal size Ki67-stained nuclei. D) p53hom/ERCC1hom liver. Arrow points towards a normal size Ki67-stained nucleus. Star is situated next to a Ki67-stained polyploid (giant) nucleus and eight-point star is situated next to a Ki67-stained polyploid binucleate cell.



(a) Wild type nucleus



(b) Big (polyploid) ERCC1 null nucleus



(c) Small (diploid) ERCC1 null nucleus

Figure 3.12: Centromere staining in wild type (a), polyploid null (b) and diploid null (c) hepatocytes. NR antibody was visualised by Texas Red. Each centromeric structure is represented by a red spot. The DNA is stained with DAPI and presents a blue color. The majority of the centromeric structures in (a) are single spots so this nucleus is considered to be in G1. On the other hand the majority of the red dots in (b) and (c) are double spots so these nuclei are considered to be in G2.

70% of the wild type hepatocytes examined. Only 30% of the wild type hepatocytes examined were at G2 (identified by a prominence of double centromeric structures). In the case of ERCC1 null hepatocytes belonging to an age-matched littermate, approx. 75% of the hepatocytes were in G2. Figs. 3.12(b) and 3.12(c) represent such nuclei. It

is noteworthy that both diploid (small) and polyploid (big) nuclei were found to be at G2 as shown by a majority of doubled centromeric structures in these nuclei. This fact reinforces the suggestion presented in the previous section that the G2 arrest seen in the ERCC1-deficient hepatocytes is a phenomenon that affects the general hepatocyte population and not the polyploid cells only.

It is now possible to answer the question that was presented in the previous section regarding the distribution of cell cycle phases in ERCC1-deficient hepatocytes. It follows from the results obtained with the centromeric staining studies that amongst the different populations of cells (G1, G2 and M) that could be responsible for the increased ratio of (G1+G2+M) to S cells in the ERCC1-deficient hepatocytes (see previous section, Fig 3.10, Table 3.1), the most likely to be responsible, given its high incidence amongst the ERCC1-deficient cells, are cells arrested in G2.

It would have been desirable to have performed this technique on double null hepatocytes. The prediction for these cells would be that since they escape the G2 arrest, they would not show the same pattern of centromeric staining as the ERCC1 null hepatocytes. Thus, we would be most likely to see the single-dot pattern seen in the wild-type cells. Unfortunately, this could not be done due to the lack of suitable material. This technique is carried out using fresh tissue and it was not possible to obtain any double null live animals before the conclusion of this thesis. In an attempt to overcome this obstacle, centromeric staining of fixed material was attempted with no positive results. This particular experiment awaits the production of more double null stock to be suitably completed. This experiment will be carried out in David Melton's laboratory.

3.2.7 p21 is a possible mediator of the G2 arrest seen in ERCC1 null livers

p21 is involved in cell cycle arrest at the G1 checkpoint of the cell cycle as part of a p53-dependent pathway (Bates and Vousden, 1996). p21 null transgenic mice are viable, showing that p21 is not essential for development. They do not show the increased incidence of tumours associated with p53 absence. Fibroblasts from these animals, however, cannot arrest in G1 following DNA damage as expected (Brugarolas

et al., 1995; Deng et al., 1995), and what is more, human diploid fibroblasts and human tumour cell lines deficient in p21 show similar characteristics (Waldman et al., 1995; Polyak et al., 1996; Brown et al., 1997).

From the studies of the latter cells another role for p21 has emerged. p21 has also been involved in both p53-mediated G2 arrest and coupling of DNA synthesis and mitosis (Waldman et al., 1996; Polyak et al., 1996). This notion has also been supported by the findings in another transgenic mouse model (Wu et al., 1996). According to Wu and collaborators, mice overexpressing p21 in their liver present a liver phenotype and developmental characteristics very similar to the ones found in ERCC1-deficient mice. In his paper Wu proposes that a likely explanation for the phenotype he observes is the p21-mediated arrest of the hepatocyte cell cycle most probably at G2.

To see if elevated levels of p21 could be associated with the cell cycle arrest seen in ERCC1 null mice, total RNA extracted from the four different types of liver was assayed for p21 mRNA levels. The graph below shows the overall results obtained from the northern analysis (Fig. 3.14). Pooled results from the analysis of a total of 11 non-runted wild type animals, 26 ERCC1-deficient animals, 3 non-runted p53-deficient animals, and 3 double null animals are represented in this figure. The values represented in this graph correspond to the average p21 mRNA levels (expressed as p21/GAPDH signal values) corresponding to each phenotypic class. Figure 3.13 corresponds to a sample northern autoradiography of several liver mRNA's probed with both p21 and GAPDH.

A number of conclusions can be derived from this experiment. Firstly, the basal level of p21 message in ERCC1^{-/-} and p53^{-/-}ERCC1^{-/-} is double and 4.5 times that of the wild type livers, respectively. Weeda and co-workers also report elevated levels of p21 in their ERCC1 k/o mouse model (Weeda et al., 1997). The fact that those are the two classes of liver tissue that present the aberrant polyploid phenotype (similar to that reported in Wu's paper) indicates that p21 may indeed play an important role in the polyploidisation process as suggested by Wu *et al.* Secondly, the level of p21 transcript in p53^{-/-} is equivalent to that of the wild type samples. This aspect has interesting implications since the expression of p21 has been extensively reported to depend mainly on its transcriptional activation by p53. There are studies, however,

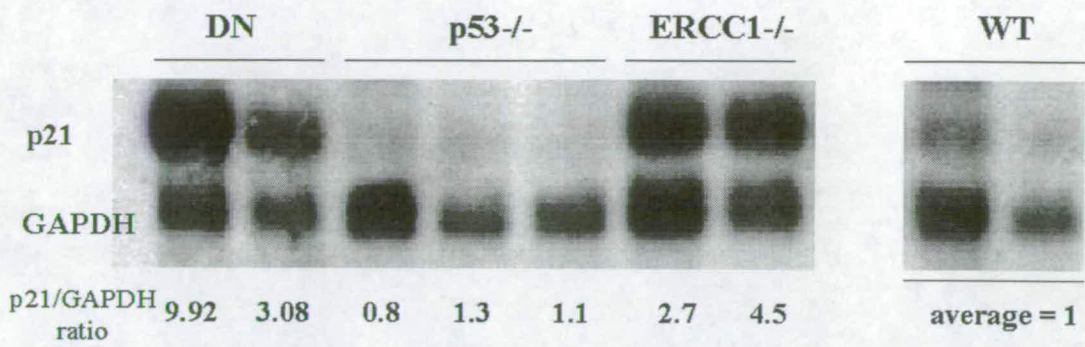


Figure 3.13: p21 northern autoradiography of total mRNA extracted from livers corresponding to the four different genotypes. All livers were extracted from 3 week-old animals. In order to obtain a loading control, the northern was reprobed with GAPDH and the ratio of p21/GAPDH signals was calculated using a phosphorimage analysis system. DN: double null; p53-/-: p53-/-ERCC1+/+; ERCC1-/-: p53+/+ERCC1-/-; WT: wild type

that show that p21 activation can also be p53-independent (Wu and Levine, 1997; Michieli et al., 1994; Cox, 1997). In support of this latter view this study shows that in mouse liver, *in vivo* expression of p21 both in the presence and in the absence of proficient NER, is p53-independent.

3.3 Discussion

There is extensive evidence that the tumour suppressor p53 has a prominent role in cell cycle regulation and apoptosis in response to DNA damage (Dumaz et al., 1998; Haapajarvi et al., 1997; Kuwano et al., 1997; Reinke and Lozano, 1997; Selter and Montenarh, 1994; Smith et al., 1995; Sun et al., 1995; Wim et al., 1997; Wu et al., 1997; Ziegler et al., 1994). There is growing evidence that supports the notion that a tight regulation of the DNA repair process and cell cycle progression is necessary in order for the cell to cope with genotoxic stress (Brugarolas et al., 1995; Kaufmann and Paules, 1996; Murray, 1994; Pellegata et al., 1996) and p21 (a major p53 effector) has been shown to play an important role in this process via p53-dependent and independent pathways (Chin et al., 1997; Kuwano et al., 1997; Li et al., 1994b; Li et al., 1996; McDonald et al., 1996; Sheikh et al., 1997). There is also growing evidence that p21 plays an active role in the DNA repair process either directly or indirectly through

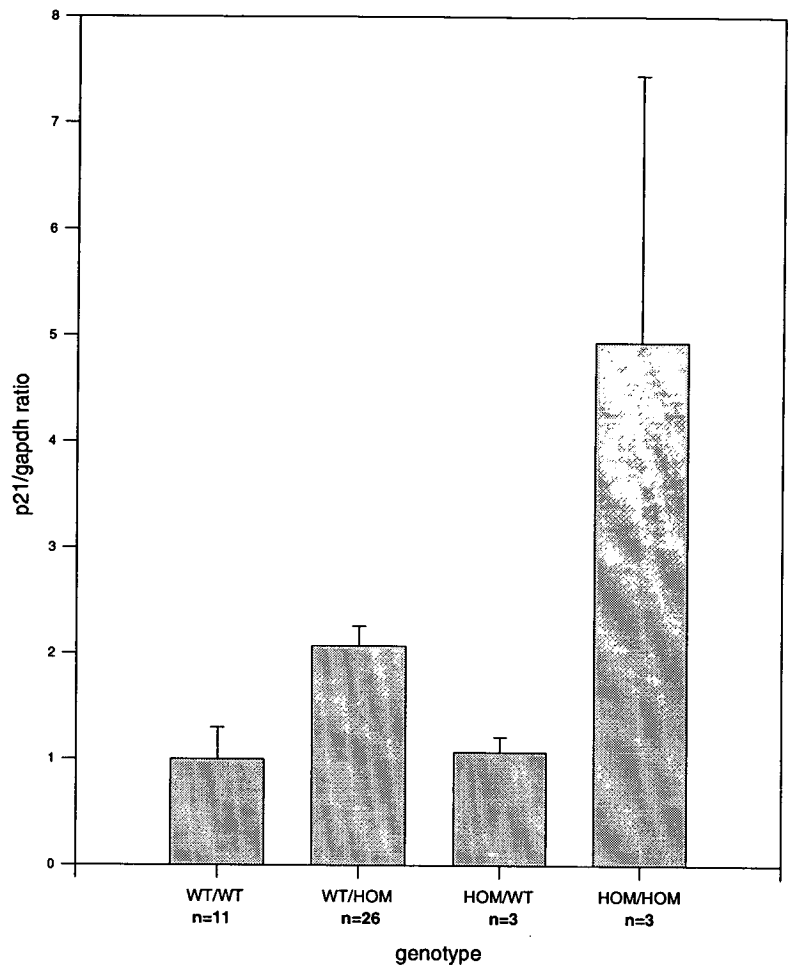


Figure 3.14: p21 levels in livers corresponding to the four different genotypes. All livers were extracted from 3 week-old animals. The bars show the means of the p21/GAPDH ratio for all the samples of a given genotype. Standard errors are shown. wt/wt stands for p53wt/ERCC1wt; wt/hom stands for p53wt/ERCC1hom; hom/wt stands for p53hom/ERCC1wt and hom/hom stands for p53hom/ERCC1hom. n represents the number of animals sampled per genotype.

its interaction with PCNA (proliferating cell nuclear antigen) (Nakanishi et al., 1995; Savio et al., 1996; Zhang et al., 1994).

The liver phenotype presented by the ERCC1-deficient mouse is a reflection of a profound misregulation of the cell cycle in ERCC1 null liver cells (McWhir et al., 1993a; Weeda et al., 1997). The fact that p53 levels had already been found to be elevated in ERCC1-deficient livers (McWhir et al., 1993a) makes the ERCC1-deficient mouse

a good model for the *in vivo* study of the relationship between p53, DNA-repair and cell cycle control. Weeda et al. also report deficiencies in the liver and spleen of their ERCC1 k/o mouse that we have not been able to see in our mice (see Conclusion section ?? for an extended discussion on this subject).

Although the liver phenotype shown by the ERCC1 null mice is very severe, the absence of just one copy of this gene seems to have no effect on the mouse liver development. Indeed, ERCC1 +/- aged mice (15-31 months) present an aged wild type-like distribution of hepatocyte nuclear area. This distribution coincides with that of young 3 week old ERCC1-deficient livers and adds proof to the idea that the phenotype presented by the young ERCC1-deficient mice is related somehow to premature ageing and severe cell cycle control misregulation.

One way of looking at this was to carry out a systematic analysis of different aspects of the hepatocytes life cycle, such as polyploidisation and binucleation rates, which vary at different times of liver development and differentiate young from old livers, in combination with cell cycle regulation. In order to do this a number of mouse strains were established in collaboration with Dr. Alan Clarke (Department of Pathology, Edinburgh University). ERCC1 and p53 were chosen to be studied in conjunction and the genotypic classes used in this study were p53+/+ERCC1-/-, p53-/-ERCC1+/+, p53-/-ERCC1-/- and wild type mice.

Binucleation

When binucleation was studied, it was clear that the binucleation process in ERCC1 deficient animals was impaired. ERCC1-/- deficient livers show a reduced percentage of binucleate cells compared to wild type. In contrast, wild type levels of binucleation are observed when p53 is not present in the ERCC1 null livers, which points towards a role for p53 in preventing binucleation when DNA repair does not occur in these cells.

Under normal circumstances, however, p53 absence does not seem to affect binucleation (see Fig. 3.4). This p53-dependent repression of binucleation in NER-defective cells makes sense in the context of the role of p53 as 'guardian of the genome'. It is not desirable for cells that cannot repair their DNA to proceed through replication (or binucleation, which involves a round of replication followed by an acytokinetic cell

division) in the presence of damaged DNA. This could lead to the perpetuation of possible lethal and carcinogenic mutations and the creation of chromosomal aberrations. p53 plays a crucial role in preventing this type of situation from occurring by arresting cells at G1/S before they replicate the damaged DNA. In the case of ERCC1-deficient cells, p53 would be playing a similar role, this time arresting the cells at G2, thus preventing the cells from going through mitosis and from becoming binucleated in the absence of a proficient DNA repair system.

BrdU labelling index

The effect of p53 absence can also be seen at the S cell cycle point as derived from the BrdU labelling data. All runt animals present lower percentages of cells in S phase than non-runt wild type animals which would be consistent with their intrinsic growth retardation. The values obtained for normal wild type animals are in agreement with results obtained by other groups (Soames et al., 1994; Sakata et al., 1996). These low runt-related levels in the ERCC1-deficient animals are rescued back to normal, non-runt levels in the absence of p53, which again indicates a possible involvement of p53 in the control of progression through the G2 checkpoint. When p53 is not present, this checkpoint is not functional and the cells are allowed to go through additional rounds of replication, which would explain the higher levels of BrdU labelling in the double null livers.

Ratio of (G1+G2+M) cells to S cells

The difference seen in the regulation of the cell cycle in the four different types of hepatocytes is also reflected in the ratios of hepatocytes at different stages of the cell cycle in the different livers. Using both the BrdU- and the Ki67-staining results, it was possible to determine which percentage of the hepatocyte population was in either G1, G2 or M and which percentage was in S. Not surprisingly, the (G1+G2+M) to S ratio in p53 deficient cells was slightly elevated compared to the normal, wild type ratio (4 and 3, respectively), which reflects the loss of cell cycle control due to the absence of p53 in these hepatocytes.

This ratio was more highly elevated in ERCC1-deficient livers (ratio=6), but was close to normal in double null hepatocytes (ratio=2.5). It is possible that the elevation in

the pool of cells in either G1, G2 or M in ERCC1-deficient livers is due to the accumulation of G2 cells. Support for this view comes from the centromeric staining experiments discussed in previous sections. The logical conclusion regarding the situation in the double null livers is that, since these cells lack p53 and therefore bypass the G2 checkpoint, their G1+G2+M to S ratio would be close to the one seen in wild type hepatocytes. This is indeed the case. Double null livers have a G1+G2+M to S ratio of 2.5 compared to a ratio of 3 in wild types. Unfortunately, direct proof of the bypass of G2 arrest in double null cells using the centromeric staining technique could not be obtained. The expectation for the double null hepatocytes would be that they present the same pattern of centromeric staining as the wild type hepatocytes. This experiment will be carried out as soon as more double null mice are available.

Despite all this, in the present work it has also been shown that, except for the binucleation rate, the phenotype of the ERCC1-/- liver is not rescued by the absence of p53. It is possible that the increase in p53 protein levels seen in these cells is a response to endogenous damage but this p53 accumulation in itself is not an essential event in the process leading to the observed overall phenotype in terms of aberrant hepatocyte morphology and ploidy levels. It is more likely that a p53-independent pathway is involved in this process.

p21 in liver

A likely candidate to take part in such a pathway is p21. p21 has an already well established role in cell-cycle control and cell growth and overexpression of p21 in liver has also been linked to an ERCC1 null-like phenotype (Wu et al., 1996). Another line of evidence comes from studies performed *in vitro* using a human colorectal carcinoma cell line (DLD1). In this study a p53-independent role for p21 in cell growth and DNA repair (Sheikh et al., 1997) has been proposed. It is interesting to note that although the majority of the DLD1 cells overexpressing p21 grew slower and did not exhibit an apoptotic phenotype, a subset of these cells did undergo apoptotic death after becoming giant cells.

The phenotype of the ERCC1-/- and double null livers is reminiscent of the one described for the DLD1 cells upon induction of p21. Whereas human cancerous cells *in*

vitro are committed to an apoptotic pathway if they become giant cells in response to p21-dependent growth inhibitory signals, mouse hepatocytes *in vivo* may arrest their progression through the cell cycle instead. The ERCC1 null mouse may be a useful tool in the understanding of the effect of p21-mediated growth arrest and its relationship with the DNA repair machinery *in vivo*.

The mechanism by which p21 exerts its role, however, remains to be elucidated. p21 has been shown to play a role in the control of the initiation of mitosis (G2/M transition) through its effect on cdk2 and cdk1-cyclin B (Guadagno and Newport, 1996). p21 has also been shown to bind and inhibit cdk1-cyclin A complexes, which play an important regulatory role in early mitosis (G2) (Basi and Draetta, 1995). Elevated levels of p21 may be responsible for arresting the cell cycle progression in those hepatocytes that are deficient in ERCC1 at very early stages of the mouse development (for a possible additional role of p21 in double null livers, see below). Further experiments in this direction are required to fully elucidate the exact mechanism of p21 action in relation to growth control in mouse hepatocytes.

Another aspect of these ERCC1 null livers is their elevated levels of polyploidisation at an early age. Polyploidisation of hepatocytes has a strong correlation with age and the levels of toxicity (either endogenous or exogenous damage) that the liver is subjected to. Because hepatocytes absorb some toxic products as part of their function, it is possible that liver cells accumulate genetic damage more rapidly than cells from other tissues. The absence of a special pool of non-differentiated stem cells able to produce more new hepatocytes makes the polyploidisation probably a safer way to compensate for cellular losses than mitosis which may result in a high incidence of chromosomal abnormalities (Medvedev, 1986). Somatic polyploidisation in liver and other tissues has been proposed to be a form of 'physiological regeneration' and this model for polyploidisation correlates well with the inverse correlation seen between mitotic index in liver and the level of polyploidisation found in different species. Mouse liver for example, has a much lower mitotic index than rat liver and accordingly, mouse liver is the one that presents the highest level of polyploidisation.

Apart from its role in protecting from the deleterious cellular effects of endogenous DNA damage, polyploidisation makes it possible for the liver to preserve its normal size

and the approximate volume of its functional capability without a risk of accumulation of a large proportion of aberrant, aneuploid cells, which may increase the level of cellular transformation. Nevertheless, the fact that polyploidisation starts at very early stages of the development of the mammalian liver (after birth) and at points of active growth, points towards a protective role of polyploidisation. Thus, this process would reduce the occurrence of mitoses which represent the most sensitive stage of the cell cycle and would decrease the accumulation of aberrant genomes, rather than providing an alternative way of repair and regeneration (Medvedev, 1986).

There are several hypotheses that try to explain the possible mechanism by which polyploidisation exerts its protective role.

1. By creation of redundant genetic information and reiteration of vital genes.
2. By making the cell more resistant to toxins and carcinogens.
3. By providing a morphogenetic strategy against ageing in specialised diploid systems. However, the polyploidisation of hepatocytes in later periods of life, particularly the progressive polyploidisation which is observed in mouse liver during senescence by formation of nuclei up to 32C in DNA content, is apparently an integral part of the ageing process, and more than representing a real protection against ageing it could well be a tissue-specific adaptation to the age-related cellular loss (Medvedev, 1986).
4. By reducing the deleterious effects of chromosomal aberrations which might have higher frequency in liver resulting from its detoxification functions.

The mechanism by which polyploidisation comes about involves continuous rounds of DNA replication in the absence of mitosis (a process known as endoreduplication). p21 has also been reported to be involved in the regulation of the checkpoints which prevent endoreduplication, both using p21 null cells and p21 expressing cells lacking pRb *in vitro* (Bates et al., 1998; Niculescu III et al., 1998; Waldman et al., 1996). In this study endoreduplication is observed in the presence of elevated levels of p21, as opposed to its absence. The absence or presence of pRb has not been tested in these animals but there is no reason to believe that pRb would be modified or absent in otherwise

normal ERCC1-deficient cells. This possibility, however, can easily be tested in future experiments. It is proposed therefore that misregulation of the expression of p21 (either its absence or its overexpression) results in the loss of the 'knowledge' by the affected cell that it is in G2 (see 'G2 memory', Chapter 1, section 1.6) and this leads to repeated rounds of replication in the absence of mitosis.

The other possibility is that elevated levels of p21, in response to non-repaired, accumulating damage, lead the hepatocytes towards a terminal (and also protective) differentiation pathway. p21 has been reported to play an important part in the differentiation process of several cell types (Elbendary et al., 1994; Jiang et al., 1994; Parker et al., 1995; Zhang et al., 1995). Since polyploidisation is the natural state of 'aged' hepatocytes the ERCC1 null phenotype could therefore be reminiscent of a premature ageing syndrome. This hypothesis will be tested in the context of a human premature ageing syndrome in Chapter 6.

In summary, in the present *in vivo* study, in the absence of a proficient repair system (as happens when ERCC1 is not present), endogenous damage is accumulated fast in the liver which prompts the accumulation of p53 and p21. In this system, the induction of p21 seems to be p53-independent. p53 is likely to be involved in the control of the arrest that in hepatocytes occurs at the G2 checkpoint in the absence of proficient NER. When p53 is present, the G2 checkpoint is active and hepatocytes lacking ERCC1 are arrested at that point of their cell cycle. When neither ERCC1 nor p53 are present, as in the case of double null hepatocytes, cells apparently lack the G2 checkpoint and as a consequence they go through additional rounds of replication, as shown by the normal wild-type levels of BrdU incorporation in double null livers. Whether p53 is present or not, however, makes no difference as to the final effect of the NER absence in liver and hepatocytes undergo polyploidisation regardless of the p53 status.

p21 may also have an active role in a p53-independent manner in the regulation of the G2/M checkpoint control, promoting G2 arrest, possibly after a previous deregulation of the S to G2/M transition, or/and in senescence. It is noteworthy that in double null hepatocytes, where p53 is not present and p21 mRNA levels are elevated, the arrest at the G2 checkpoint is nevertheless bypassed. This may suggest that the main role of p21 in this particular system could be more likely related to the regulation of hepatocyte

differentiation and senescence.

Hepatocytes lacking NER are more prone to accumulation of endogenous damage. The way this type of cell deals with this situation is by promoting polyploidisation, a process that involves both p53, in a manner that appears to be directly linked to cell cycle regulation at the G2 checkpoint, and p21, which in a p53-independent fashion (may be playing a more prominent role in the absence of p53) would probably promote the terminal differentiation of these cells. Both the p53-related and the p21-related mechanisms would have as an overall result the premature hepatic polyploidisation process seen in the ERCC1-deficient livers and their aged appearance.

Chapter 4

In Vitro Studies: Effect of UV on Replication in ERCC1-Deficient Fibroblasts

4.1 Introduction

From the studies of the morphology and the cycling characteristics of the ERCC1-deficient hepatocytes, it was clear that the absence of ERCC1 severely affects the regulation of the cell cycle in these cells. In the previous chapter it is argued that ERCC1-deficient hepatocytes bypass the DNA damage-inducible G1/S checkpoint control and that they are subsequently arrested in G2 only if p53 is present. It was thus of interest to study how ERCC1 null cells behave in terms of replication under conditions of damage. If the normal G1/S checkpoint control is overridden in these cells under conditions of endogenous damage, does this mean that the expected arrest after UV damage is also affected? Do ERCC1-deficient cells show a different response to UV light compared to their wild type counterparts?

This chapter deals with the experimental approach undertaken in an attempt to answer those questions. Therefore, in this chapter special attention is paid to the mechanisms of replication arrest under conditions of UV damage in excision repair-deficient and -proficient rodent cells. Extensive work has been carried out regarding this subject (for a review see Meneghini *et al.*, 1981 and the Chapter 1, section 1.2 in this thesis), but it is not clear yet what the mechanisms of DNA inhibition by pyrimidine dimers are and what exactly happens in terms of the recovery from DNA synthesis arrest after

UV irradiation.

It is known that after UV irradiation DNA synthesis is arrested and that after a certain period of time, it resumes again and replication proceeds as usual. Experiments carried out with human XP cells (deficient in NER) show that such recovery in DNA synthesis does not happen in the absence of NER (Lehmann, 1979). However, it is also known that NER is not enough to bring about such recovery, as is the case for human fibroblasts derived from patients with Cockayne's syndrome. These cells do not show recovery of DNA synthesis after UV but nevertheless they are able to carry out excision repair of dimers (Lehmann et al., 1979). The exact mechanisms behind this recovery are unknown, but there is a hint that it happens during S phase and protein synthesis is necessary for it to occur in human fibroblasts, as shown by experiments performed with protein and DNA synthesis inhibitors (Lehmann, 1979). Differences between rodent and human cells arise at this point. CHO cells seem to require DNA synthesis (passage through an S phase) to bypass the replication arrest (Meyn et al., 1977). Also, only 25% of the dimers are eliminated in CHO cells after 5 J/m^2 , which seems to indicate that NER does not seem to account fully for the recovery in rodents (Meneghini et al., 1981).

It has been suggested that the DNA recovery mechanism may be induced when DNA synthesis is inhibited. Specific proteins would be synthesised and a more effective DNA synthesis would be directed to the damaged template. This inducible process would resemble the bacterial SOS response (Meyn et al., 1977). It is also possible that the recovery mechanism is composed of NER and an inducible process dependent on *de novo* protein synthesis. This model would account for the behaviour of normal, XP variant, XP and rodent cells. The latter ones would be more dependent on the inducible process whereas the human cells would rely mainly on the NER mechanism (Meyn et al., 1977; Dahle et al., 1980).

We wanted to study the behaviour of ERCC1-deficient rodent cells in terms of their capacity to recover from DNA synthesis arrest. In order to do so we used a method (described in the Materials and Methods, Chapter 2, section 2.2.12), that relies on the study of the number of double-strand breaks (DSBs) that can be detected after different periods of time post-UV irradiation. Briefly, a primary lesion in the DNA

(a single strand break) is converted to a DSB in the arrested replication fork by the enzymatic action of DenV and a decrease in the number of DenV-induced double strand breaks after UV irradiation is taken as an indication of the recovery from DNA synthesis arrest (fork progression arrest). Overall, the experimental procedure presented here allows us to correlate the rate of recovery from replication arrest with the extent of repair in the cell.

In these experiments we used both CHO and transformed mouse cells. The choice of transformed cell lines was determined by the experimental procedures carried out, which involve great number of cells per experiment. Unfortunately, primary fibroblasts are difficult to grow and it was not possible to carry out all experiments with them. The preliminary results obtained with the transformed cell lines prompted the necessity to investigate further an aspect of DNA synthesis recovery that appeared to be different in ERCC1-deficient and NER proficient transformed cells. It was of vital importance then to find an experimental procedure that would allow a more flexible handling of the cells which if necessary, could also be used with primary fibroblast cell lines.

The first part of this chapter deals with the former set of experiments up to the point where an apparent difference in recovery of DNA synthesis was identified (see section below, Theory). These experiments were carried out in Robert Johnson's laboratory in Cambridge under the supervision of Robert Johnson and Shoshana Squires (CRC Unit, Zoology Department, Cambridge). The rest of the experimental procedure was devised and carried out in Edinburgh and the results are presented in the second part of this chapter.

The cell lines used for the experiments are CHO9, CHO 43-3B (parental wild type transformed Chinese hamster cell line and ERCC1-mutant transformed Chinese hamster cell line respectively), CHO AA8, CHO UV5 (parental wild type transformed Chinese hamster cell line and ERCC2-mutant transformed Chinese hamster cell line, respectively), PF20, PF24 (wild type transformed mouse cell line and ERCC1-mutant transformed mouse cell line, respectively) and PF+/+ and PF-/- (wild type and ERCC1-deficient primary fibroblasts, respectively). All the CHO lines were kindly provided by R. Johnson.

Another aspect we wanted to investigate was the relationship between a possible misregulation of the repair and the DNA synthesis processes in ERCC1-deficient cells that would explain some of the phenotypic characteristics observed in the ERCC1-deficient mice. It was clear from the morphology of the ERCC1 null livers (polyploidy, aneuploidy, aged appearance) that the cause of this phenotype had to do with cell cycle regulation defects. Since ERCC1 and replication protein A are known to interact and replication and DNA repair seem to have a tight interaction there was a possibility that the mutant phenotype was related to an ERCC1-dependent misregulation of DNA replication.

Studies of the relationship between DNA repair and replication of DNA have shown that the precise coordination of these two processes under genotoxic stress is of vital importance for the survival of the cell. Several genes and their corresponding products have been characterised as playing a role in this coordination process from the replication, the cell cycle and the DNA repair machineries.

A key player in this process is PCNA (Jonsson et al., 1998). p21 is also known to interact with PCNA (Li et al., 1994b) and to have an inhibitory effect on DNA replication (Chen et al., 1996a; Chen et al., 1996b; Jackson et al., 1995). Another protein that has been involved in the interaction between cell cycle control and replication is DNA polymerase ϵ (Navas et al., 1995). RPA (replication protein A), a well characterised replication protein has a role both in replication and DNA repair and more specifically it has been characterised as conferring specificity to the ERCC1/XPF endonuclease activity (Matsunaga et al., 1996).

The interaction between the above mentioned proteins indicates a fine balance and a careful and tightly regulated mechanism by which, under conditions of DNA damage, the cell cycle is arrested at the G1/S checkpoint, before the onset of replication, so that the repair mechanisms can repair the damaged DNA before replication takes place and the damage is perpetuated. Another checkpoint at which arrest can take place is the G2 checkpoint. In this case, cells arrest entry into mitosis before damaged DNA is passed on to the daughter cells. This latter control mechanism is studied elsewhere in this thesis (Chapter 3).

Results obtained using the experimental procedures described here and experiments carried out by Melton and collaborators (Melton et al., 1998), have allowed the construction of a model that explains the phenotype observed in these mice in terms of the way they deal with DNA damage and how they regulate the control of the cell cycle under conditions of UV damage.

4.2 Theory

4.2.1 Repair of cyclobutane pyrimidine dimers (CPDs)

In order to study the removal of CPDs from the DNA replicating at the time of UV-irradiation ($[^3\text{H}]$ -labelled) and from the rest of the genome ($[^{14}\text{C}]$ -labelled), analysis of DenV-induced SSBs in DNA extracted from these cells was carried out using alkaline gel electrophoresis. It is assumed that DenV cuts at all dimer sites present in the DNA. DenV incises at a dimer to give a single strand DNA break (Enzyme-sensitive site, ESS). Thus, the number of SSBs generated by the DenV action represents the number of incisions at CPD sites. It follows that the disappearance of ESS with incubation indicates the removal of dimers by the cellular repair machinery.

Replicating DNA and bulk DNA can be differentially labelled with $[^3\text{H}]$ TdR and $[^{14}\text{C}]$ TdR respectively. Once labelling is done, cells are irradiated and allowed to recover after injury. By plotting the number of remaining ESSs in $[^3\text{H}]$ TdR and $[^{14}\text{C}]$ TdR labelled DNA after the recovery time, the rate of repair in replicating and in bulk DNA can be deduced.

The number of single strand breaks (SSBs) per unit DNA are calculated from the migration profiles of the alkaline gels, as stated in Materials and Methods, section 2.2.12. The ESSs are defined as the breaks produced by the DenV acting on the UV-irradiated DNA.

ESS = (Breaks per Mb induced by the DenV acting on UV^+ (UV irradiated) DNA) – (Breaks per Mb generated by the DenV acting on the unirradiated control).

4.2.2 Arrest of the replication machinery at the UV-induced dimers and the time course of the recovery from the stalling.

These parameters can be studied from the results obtained from the pulse field gels (AFIGE) (Denko et al., 1989; Stamato and Denko, 1990). The presence of a single strand gap in the daughter strand opposite a pyrimidine dimer can be identified by the generation of Double Strand Breaks (DSBs) when the DNA embedded in agarose plugs is treated with DenV (see Materials and Methods, section 2.2.12).

In this procedure, the amount of DNA that enters the gel is a measure of the number of DSBs formed and it is determined by calculating the fraction of radioactive counts that are released into the gels (any DNA fragment less than 1.6 Mbp long is released into the gel) as a percentage of the total radioactivity (%FAR) in that sample.

It has been shown that the amount of DNA released into the gel varies linearly with the X-ray dose for both bulk and pulse-labelled nascent DNA, but the FAR values for the replicating DNA (^3H -labelled) are several times lower than those of the bulk DNA (^{14}C -labelled) (Stamato and Denko, 1990; Ryan et al., 1991). With the AFIGE it is not possible to calculate the number of DSBs per unit of DNA directly but it can be estimated indirectly by comparing the %FAR of the experimental DNA with that of DNA exposed to a known dose of X-rays.

The different response of the replicating and bulk DNA to a given X-ray dose varies according to the cell strain used and the time allowed after irradiation. In order to compare different samples and cell types, the results are standardised as Gy equivalent DSBs and not in %FAR. The following mathematical model is used for such calculations.

Mathematical calculations

FAR is calculated as:

$$\%FAR = \frac{\text{CPM in track for } ^3\text{H or } ^{14}\text{C} - \text{labelled DNA}}{\text{CPM in track} + \text{CPM in well (total CPM)}} \quad (4.1)$$

As γ irradiation induces both single and double strand breaks in the DNA which are equally distributed, and given the fact that the number of DSBs cannot be measured directly from the Pulse Field gels, the number of DSBs induced in the UV treated

samples is estimated from the %FAR of samples that have received a fixed dose of γ irradiation (100Gy).

In the γ irradiated samples the %FAR recorded is the sum of the release due to the fixed dose of 100Gy plus the background levels of breaks, which are generated in the cell.

For any given sample (S) and the same γ irradiated sample (G):

the %FAR due to a dose of a 100Gy is A

$$A = \%FAR \text{ in } G - \%FAR \text{ in } S \quad (4.2)$$

and therefore the DNA DSBs (Gy equivalent) are calculated as B.

$$B = 100 \times \%FAR \text{ in } S \text{ (UV or mock)} / \%FAR \text{ of } A \text{ (due to 100Gy)} \quad (4.3)$$

Cellular DSBs induced by UV irradiation are:

$$a = B \text{ of } UV^+ - B \text{ of } UV^- \quad (4.4)$$

DSBs induced after DenV treatment are:

$$b = B \text{ of } DenV^+UV^+ - B \text{ of } DenV^-UV^- \quad (4.5)$$

b is the sum of cellular DSBs(a) and those that were generated by DenV due to stalling of the replication machinery. Thus the DenV associated DSBs (ESS DSBs) are (c):

$$c(\text{Gy equivalent}) = b - a \quad (4.6)$$

4.2.3 Arrest of the replication machinery after UV irradiation: Direct Assay

In order to carry out a detailed study of the kinetics followed by the different cell types after UV it was necessary to devise a simpler assay that allowed the collection of specific data in an easy-to-analyse form. Thus, a short replication assay that allows us

to monitor the response at the level of replication in ERCC1-deficient and wild type cells after UV irradiation was devised.

This assay has three major advantages over the previous experimental set up.

- It only uses one type of radioactive isotope (tritiated thymidine).
- It allows the synchronisation of the cells by isoleucine deprivation (G1 arrest).
- It is fast and the data is easily interpreted.

Briefly, the cells are plated out at the desired density and they are allowed to settle overnight. The next day they are changed into an isoleucine-free medium for 16–18 hours to obtain a G1 arrest. They are then released into S phase by changing their medium back to complete growth medium. The cells are then irradiated in S with a given UV dose after which they are pulsed with tritiated thymidine for 15 min. The incorporation of radioactivity into their DNA is subsequently measured in a scintillation counter. All the data collected are in the form of DPM counts.

Typically, a decrease in the incorporation levels (DPMs) relates to a decrease in replication. Likewise, an increase in the levels of replication will be reflected in a bigger tritiated thymidine uptake by the cell and higher radioactivity measurements. This allows us to see if there is a bypass of the arrest of the replication process after UV damage in the ERCC1 null cells.

4.3 Results

4.3.1 ERCC1-deficient transformed fibroblasts show impaired DNA repair activity.

It has already been shown that cell lines defective in DNA repair (PF24, ERCC1-/-) are impaired in their capacity to repair DNA (Melton et al., 1998). Thus, it is logical to assume that PF24 cells cannot remove DenV-induced single-strand breaks from their genome after UV damage. In order to test this, the rate of removal of SSBs from the DNA of NER-deficient versus NER-proficient mouse transformed fibroblasts (PF24 versus PF20) was assayed using the alkaline gel electrophoresis method.

Cells were prepared and treated as described in Materials and Methods and the results obtained are shown in Figure 4.1. This graph represents the number of ESS sites detected in both the bulk and the replicating DNA extracted from these cells after UV-irradiation ($10\text{J}/\text{m}^2$). The number of ESS sites is plotted against time following UV irradiation.

PF20 (ERCC1+/+) cells show a decrease of ESS sites with time in both bulk and replicative DNA. The decrease is noticeable 30 minutes after UV (14% less ESS than immediately after UV irradiation in the replicative DNA and 26% less ESS in the bulk DNA). The rate of loss of ESS sites is not rapid in either case, but this is in agreement with the low rate of repair that rodent cells exhibit after UV irradiation (Meneghini et al., 1981). In addition, the rate of decrease seems more rapid in the replicating DNA, which suggests that in mouse cells proficient in DNA repair, this repair takes place preferentially in the replicating DNA.

As expected, PF24 cells show a different response. These cells seem to accumulate ESS sites at a higher rate than their wild type counterparts and these sites do not decrease with time below the starting level. This is likely to be a reflection of their inability to remove ESS (CPDs) after UV.

In summary, NER seems to play a prominent role in the removal of SSBs in mouse cells. This removal seems to be preferentially directed towards replicating DNA and, when it happens, it is not very pronounced, which is in accordance with already published observations (Meneghini et al., 1981).

4.3.2 NER-deficient rodent cells behave differently to NER-deficient (XPA) human cells in response to UV damage.

As mentioned before, stalling of the replicating machinery can also be studied with this experimental approach. Calculations were carried out that provided information on stalling after UV in the different cell lines studied. Figure 4.2 shows the effect of UV in the mouse cells both in replicating and bulk DNA. This response is also compared to the response shown by a human XPA deficient cell line (data kindly provided by Shoshana Squires and Robert Johnson, Cambridge).

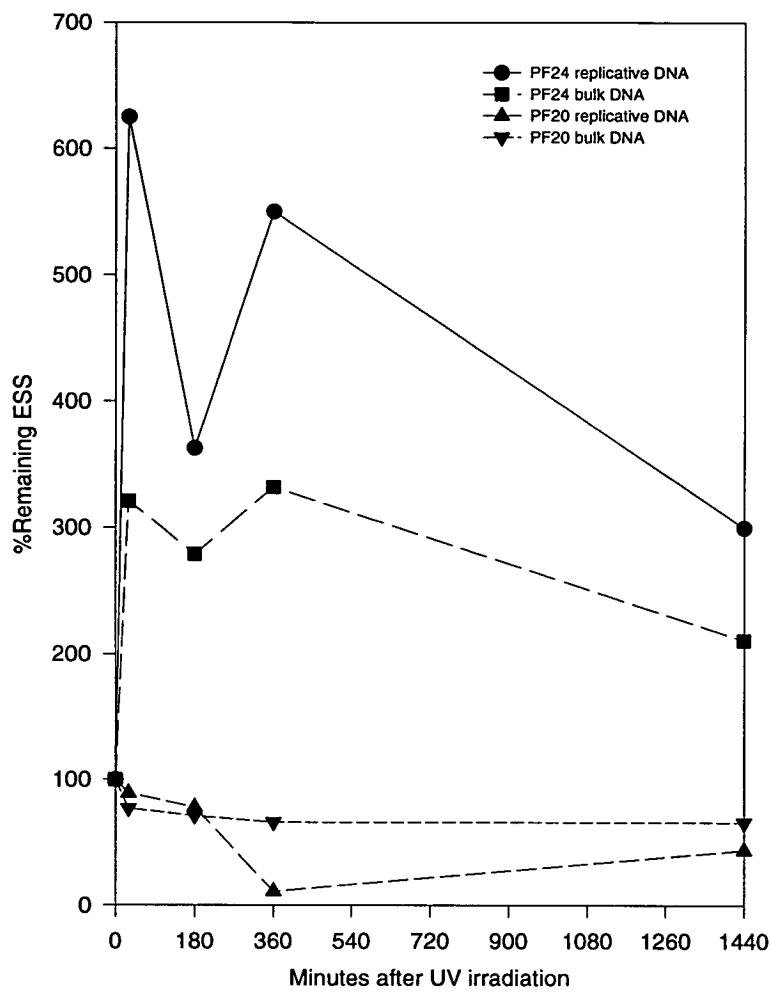


Figure 4.1: Repair capacity of PF20 (ERCC1 wild type) and PF24 (ERCC1-deficient) mouse transformed cell lines as indicated by the percentage of ESS sites remaining after UV.

Briefly, any decrease in the number of DSBs as shown in any of these graphs is a reflection of the DNA-replicating machinery proceeding with replication after UV (see Introduction and Theory sections in this chapter, as well as Chapter 1 in this thesis).

When both the replicating and the bulk DNA are studied by this method they show different behaviour in the way UV-induced damage affects them. Overall, the amount of DSBs induced in the bulk and replicating DNA differ in number (Fig. 4.2 top vs. bottom panel), which can be interpreted as replicating DNA being more sensitive to the formation of DSBs. This is an expected behaviour since replicating DNA is more

vulnerable to external damage than the protected, compact, supercoiled bulk DNA. Also, this may be an indication of the existence of gaps in the daughter strands of replicating DNA, which would make the number of DSBs higher.

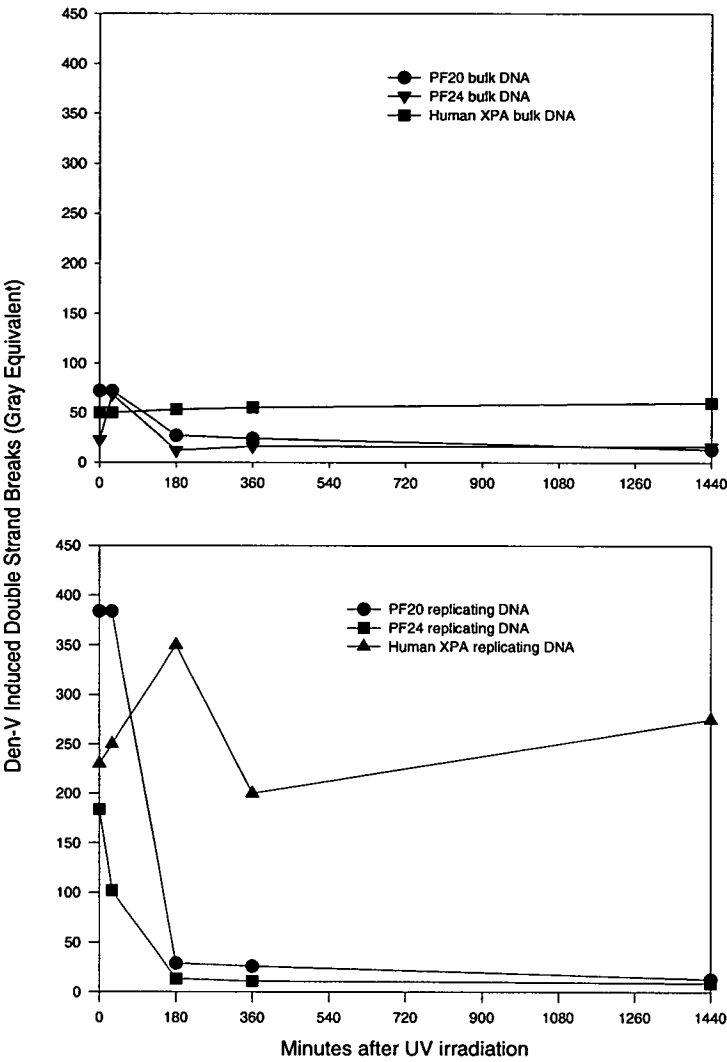


Figure 4.2: Replication stalling as measured by the presence of DSBs in PF20 and PF24 cell lines. A human cell line deficient in XPA is also shown. The top panel represents the stalling in the genome overall. The bottom panel represents the stalling in the replicating DNA.

As expected, the human cell line deficient in DNA repair, does not show a decrease in the number of DSBs after UV (both in the genome overall or the replicating DNA), which suggests that DNA synthesis remains stalled at the unrepaired lesions. This mechanism ensures that the cell does not replicate damaged and therefore potentially mutagenic DNA.

However, this is not the case for the mouse cell lines. Both PF20 and PF24, seem to continue with the replication of their DNA (shown by the rapid decrease in DSBs) regardless of the damage inflicted upon their DNA. We know that damage is not removed efficiently (if at all) in these cell lines as shown with the alkaline gel electrophoresis procedure (see previous section). PF20 cells, which are capable of DNA repair, do not seem to remove SSBs very efficiently and PF24 cells, do not seem to do this at all. These kinetics of DSB removal are also shown with CHO cells (see Fig. 4.3). The number of DenV-induced DSBs in the replicating DNA from both CHO9 and CHO 43-3B cells decline rapidly soon after UV irradiation. By 3 hours after UV the number of DenV-induced DSBs has decreased to approximately 37 % of the initial number in CHO9 cells and to approximately 15 % of the initial number in CHO 43-3B cells. Again this is an indication of rapid progression of fork movement after UV irradiation. In the case of CHO 43-3B cells this happens in the absence of lesion removal which, overall, seems to suggest that the existence of pyrimidine dimers is not an efficient blockage of fork progression in these cells and more importantly, NER does not seem to play a major role in the process of recovery from such arrest.

This behaviour is in contrast with the human behaviour and could be an intrinsic difference in the way rodents and humans deal with their damaged DNA. It is likely that human cells rely on NER for the recovery of the arrest of fork progression, whereas hamster and mouse cells possess another mechanism, different from NER, that elicits a prompt recovery despite the presence of pyrimidine dimers and possible breaks in their DNA. This is supported by the fact that hamster cells seem to behave more like the mouse cells than human cells.

There is an indication that ERCC1-deficient mouse cells (see Fig. 4.2) may arrest for a shorter time than their wild type counterparts. By 30 min. after UV radiation DSBs have already diminished to approximately 55 % of the initial number in the mutant cell line whereas DSBs remain unaltered in the PF20 wild type cells. This apparent difference between ERCC1 wild type and ERCC1-deficient cells seen with this experimental method, prompted the following simpler and more flexible experiments in which the main focus was to look in more detail into those first 30 min. after UV-irradiation with an appropriate set of NER-deficient cells and their corresponding

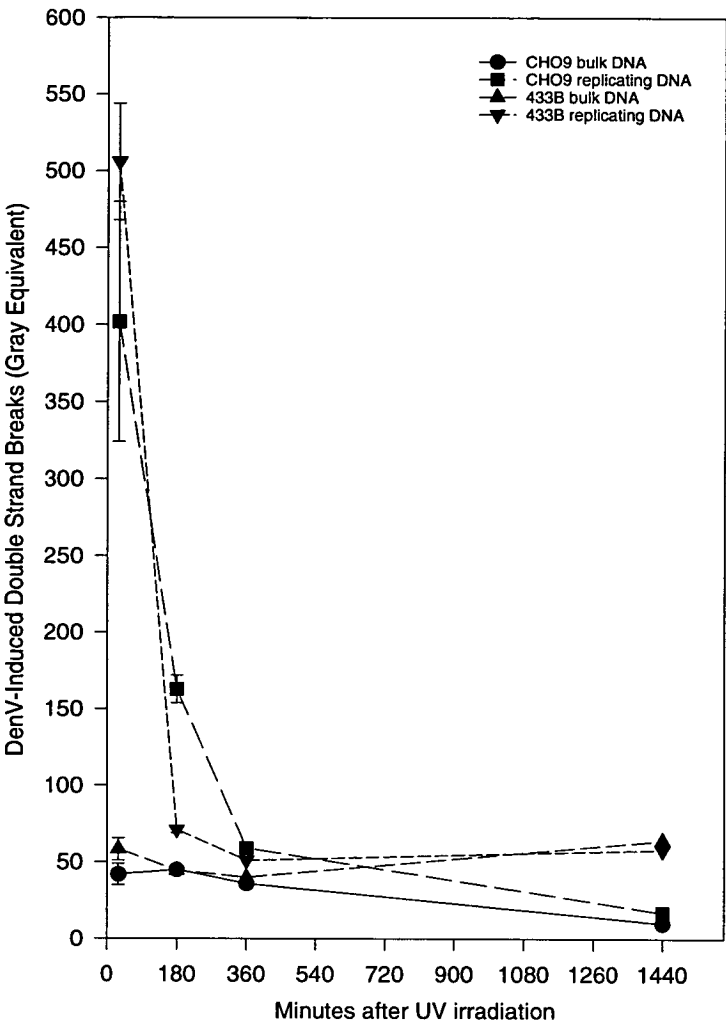


Figure 4.3: Rate of disappearance of stalled replication sites with incubation time in CHO9 (repair proficient) and CHO 43-3B (repair deficient) cell lines.

wild type controls.

4.3.3 Cells arrested in isoleucine-deprived medium and then released reach S-phase within 6 hours

The experimental rationale behind this new set of experiments was to find a simple, fast method for studying the replication response of different sets of cells after UV. In order to see how UV affected progression through replication, cells had to be synchronised and irradiated in S-phase. It was important to get a good synchronisation in order to avoid background replication noise.

Isoleucine deprivation was chosen as the blocking method. Isoleucine deprivation causes the cells to arrest at G1. It was important therefore to know when the cells reached S-phase after the release from the block. The experiment described below allowed the estimation of the time of entry into S from a G1 arrest in the cells to be studied.

A typical response curve of two transformed mouse cell lines is shown below (Fig.4.4). Cells were arrested in isoleucine-deprived medium for a minimum of 10 hours and the response in terms of tritiated thymidine uptake during short, 15 minutes, incubations in labelled medium (direct measurement of replication) was monitored at different intervals of time during their release into normal, complete medium for up to 8 hours.

At 0 hours after release, the tritiated thymidine incorporation is at a minimum (cells are arrested efficiently) and subsequently it increases steadily as the cells enter into S-phase. By 6 hours after release cells were well into S phase. These results were taken as the basis for the time course chosen for subsequent experiments (see below). The UV dose chosen was 20 J/m² in order to make sure that high damage rates were obtained and to increase the probability of obtaining a clear arrest in the replication progression in the different cells.

4.3.4 There is no difference in the DNA replication behaviour of ERCC1+/+ and ERCC1-/- mouse cells in the first two hours after UV.

As mentioned before, the previous experimental setup was very informative in terms of looking at the differential behaviour of replicating versus bulk DNA after UV damage and provided good data regarding the repair capacity of ERCC1+/+ versus ERCC1-/- deficient cells. However, given technical difficulties with the timing and work load involved in the preparation of the samples to be examined, it was impossible to use this same assay to follow replication arrest in the first 2 hours after UV with sufficient detail. This period was of interest because there was a hint that ERCC1-/- transformed fibroblasts had a shorter arrest after UV damage than ERCC1+/+ transformed fibroblasts (see section 4.3.2).

With the method presented here, the amount of handling involved was reduced considerably and it was possible to synchronise the cells in order to obtain a more accurate

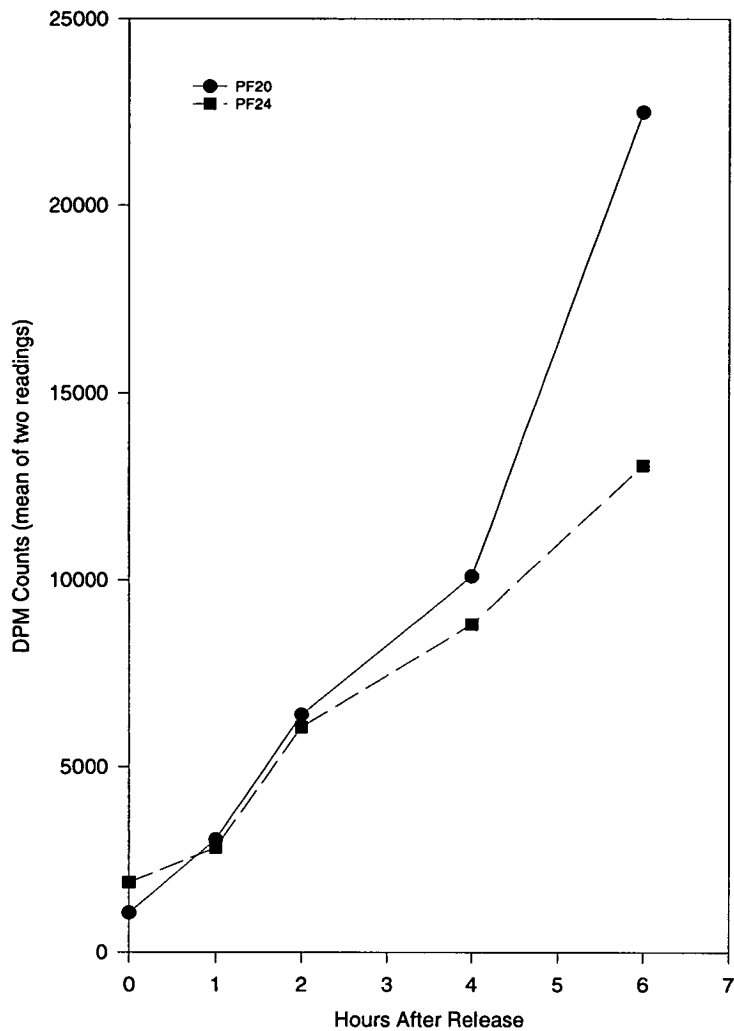


Figure 4.4: Tritiated thymidine incorporation (measured as total DPM counts) in two cultures of transformed mouse fibroblasts (PF20 and PF24) after release from an isoleucine block (10 hours).

idea of the behaviour of the transformed cell lines after UV. If the results obtained with the transformed cell lines showed a significant difference between the ERCC1-deficient and the ERCC1 wild type cell lines, this method was also flexible enough to allow working with the more sensitive primary fibroblasts cell lines.

If ERCC1-deficient cells had a shorter arrest of replication after UV it should be possible to detect this using this method. It would be expected that a shorter arrest time would result in a faster increase in the uptake of tritiated thymidine after UV by the mutant cell line. The experiment was performed with transformed mouse fi-

broblasts (PF20, PF24; ERCC1+/+ and ERCC1-/- respectively), transformed CHO cells (CHO9, CHO 43-3B; ERCC1+/+ and ERCC1-/- respectively) and also mouse primary fibroblasts (PF +/+, PF -/-; ERCC1+/+ and ERCC1-/- respectively) and the results are shown in Fig. 4.5.

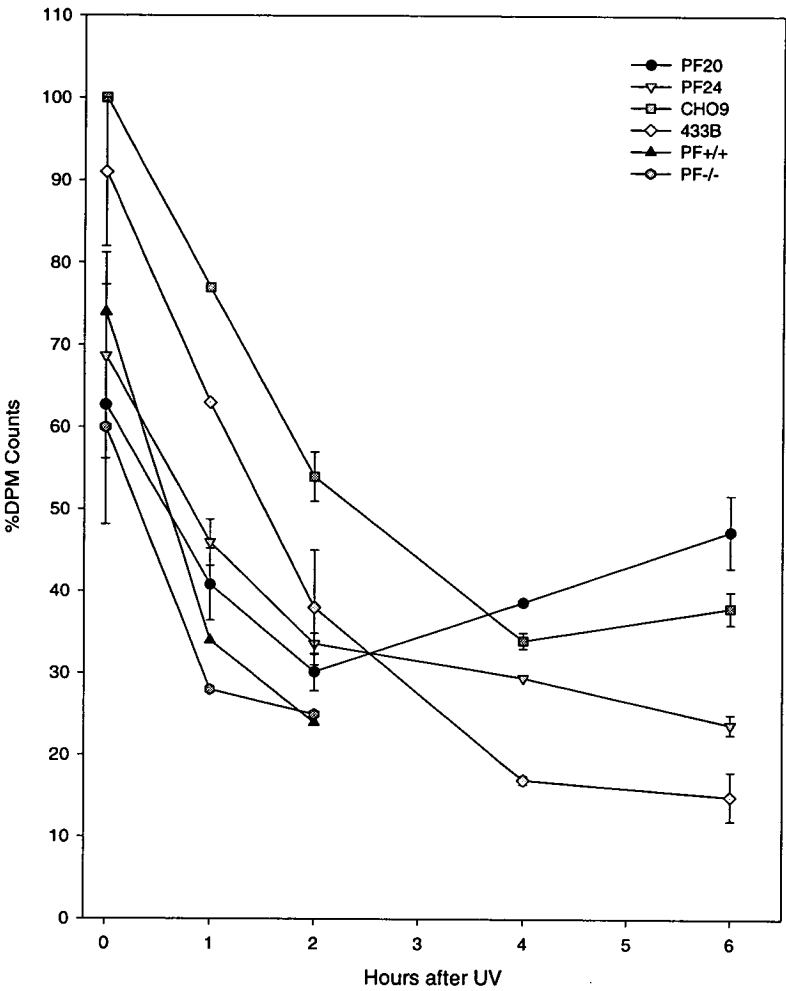


Figure 4.5: Tritiated thymidine incorporation (measured as %DPM counts relative to the mock samples) in PF20 (ERCC1+/+), PF24 (ERCC1-/-); CHO9 (ERCC1+/+), CHO 43-3B (ERCC1 mutant); PF +/+ (ERCC1+/+), PF -/- (ERCC1-/-) after release from isoleucine block (10 hours) and UV irradiation (20J/m²). Each point belonging to a single experiment represents two duplicate measurements. For the primary fibroblasts one experiment is reported. For the transformed cell lines two experiments are reported. The error bars represent the mean standard error.

This graph shows the incorporation of [³H]Thymidine by the different synchronised cell lines after a dose of 20 J/m². For the mouse primary fibroblasts only one experiment

was carried out (each point represents the average of duplicate samples) and for the transformed cell lines two separate experiments are shown (the error bars represent the mean standard error).

In order to make sure that there were no major differences in the behaviour of the primary and the transformed mouse cell lines, a preliminary, shorter, experiment was set up that has been included in this graph. The time course studied with the primary fibroblasts covers only the first two hours after UV irradiation ('critical' time). The results obtained suggest that both primary and transformed mouse fibroblasts behave in essentially the same manner. Both transformed and primary wild type mouse cells show an equivalent reduction in the [^3H]Thymidine uptake 2 hours after UV (~45 % reduction in CHO9 cells, ~48 % reduction in PF20 cells and ~65 % reduction in PF+/- cells). In addition, there is not a significant difference in the incorporation of [^3H]Thymidine at the same time point after UV between the NER-proficient and the NER-deficient cell lines (~59 % reduction in CHO 43-3B cells, ~51 % reduction in PF24 cells and ~57 % reduction in PF-/- cells) which is in accordance with the results described in the previous sections. Thus, the rest of the longer, more demanding experiments were performed with the transformed cell lines, PF20 and PF24.

It was also clear from this experiment that there was no significant differences in the trend followed by the CHO transformed cell lines, CHO9 and CHO 43-3B, when compared to their mouse counterparts, after the UV challenge. This similarity in the response of transformed CHO and mouse cell was used as the basis for the next experiment (see below).

The trend followed by all the cell lines used in this experiment is the same. They all reduce their tritiated thymidine uptake after UV irradiation as would be expected. This decrease reaches a minimum between 2 and 4 hours after UV-irradiation as previously reported for rodent cells (Meneghini et al., 1981). In order to see if this decline in the incorporation of tritiated thymidine was maintained at later time points and to investigate the possibility that other NER mutants may behave differently, further experiments were set up that are reported in the next section.

In summary, these results differ from the expectations derived from the previous studies

carried out on replication fork stalling (DenV-induced DSBs measurement after UV damage), from which there was an apparent difference in the kinetics of replication arrest recovery between NER-deficient and wild type transformed mouse fibroblasts. Under this experimental procedure there was no indication of a different response of the ERCC1-/- transformed mouse cell lines when compared to their wild type counterparts in the first minutes after irradiation. This was also confirmed by the other experiments carried out and presented in the next section.

4.3.5 The kinetics of recovery from UV irradiation-induced arrest are equivalent in cells from different ERCC complementation groups (ERCC1 and ERCC2).

This experiment was designed to investigate whether the recovery from UV irradiation-induced replication arrest differed in ERCC1 mutants when compared to wild type or mutants from another ERCC group. Four different CHO cell lines, CHO9, CHO 43-3B, CHO AA8 (wild type) and UV5 (ERCC2-deficient) were used. Chinese Hamster Ovary cells (CHO) were used mainly due to the fact that ready access to ERCC1 and ERCC2 null cell lines was available which was not the case for mouse.

The following graph shows the results of the experimental work with the above mentioned cell lines (Fig.4.6). The two mutant cell lines are deficient in DNA repair and show increased susceptibility to UV irradiation. The wild type cell lines are the corresponding parental lines for each one of the mutants. This graph shows the kinetics of replication after UV irradiation in each one of these cell lines. Each cell line was arrested at G1 in isoleucine-free medium for a period of 10 hours, released into normal medium and UV irradiated with a dose of 20 J/m². Each time point in each of the experiments was carried out in duplicate and there was also a duplicate for each corresponding mock-irradiated control. In the case of the ERCC1 cell lines, each point depicts the mean from two separate experiments with error bars. For the ERCC2 cell lines each point represents the mean of three experiments.

The Y-axis represents the tritiated thymidine incorporation (DPM counts) as a percentage of the values for the unirradiated controls. The X-axis represents the time points at which each sample was collected after UV (or mock) irradiation.

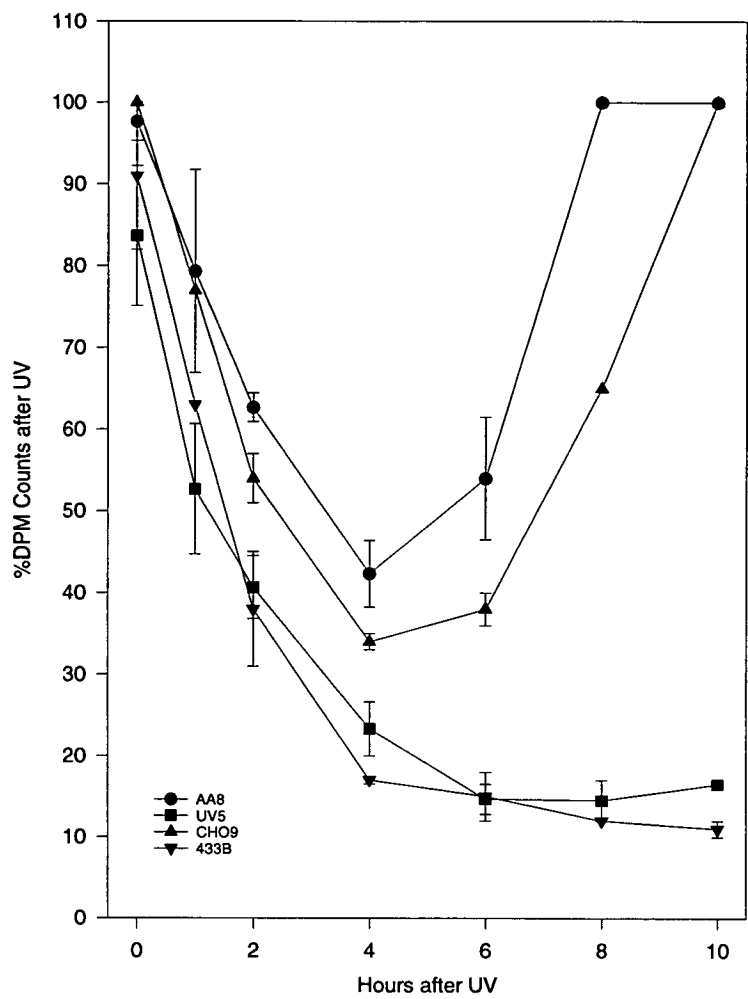


Figure 4.6: Tritiated thymidine incorporation (measured as %DPM counts of unirradiated controls) after UV irradiation in CHO9 , CHO 43-3B, CHOOA8 and UV5

There is a difference in the way parental lines behave when compared to NER deficient ones. This difference however, is not at the early time points as suggested by the previous set of experiments (Fig. 4.2), but at the later time points. Both parental lines decrease their tritiated thymidine incorporation to about 40% of controls by 4 hours after irradiation. After that, they start recovering and go back to 100% incorporation by 8-10 hours. This is in accordance with results obtained using different experimental procedures with rodent cells (Meneghini et al., 1981). The UV-sensitive cell lines show a decrease to ~20 % after 4 hours but, as would be expected after such high UV doses, do not recover after that time. In fact most cells from both cell lines, UV5 and 43-3B,

had died by 10 hours after UV irradiation.

This recovery in the rate of DNA synthesis as measured by this method seen in the NER-proficient cell lines has been reported previously for rodent cells (Meneghini et al., 1981). In our experiment, cells that are not proficient in NER (CHO 43-3B and CHO UV5) do not seem to undergo such a recovery. It is likely that NER is needed under these circumstances to overcome the DNA synthesis arrest.

This seems to be in disagreement with the results obtained in the previous sections (see section 4.3.2) in which these same NER-deficient cells seem to proceed through replication irrespective of their NER status. However, there is a major difference between these experimental procedures. The experiments described in the previous section were performed under moderate UV doses ($10\text{J}/\text{m}^2$) whereas the experimental procedure presented in this section was carried out at high fluences ($20\text{J}/\text{m}^2$). It is possible, as it has been already suggested that the mechanisms acting under these circumstances differ from one another. It could be argued that under low to medium UV doses, NER does not play an essential role in the way DNA synthesis progresses. Under these circumstances, in NER-deficient cells, fork progression (as shown by the decrease in the number of DenV-induced DSBs after UV) would proceed even in the presence of unrepaired DNA. A tolerance mechanism would be operational in these cells, different from NER (see Chapter 1, section 1.2). At high UV doses, it would be logical to assume that the damage is too big for the alternative DNA lesion-tolerance mechanism and NER would then be required to repair the damage. In this case, cells that cannot repair their DNA are not capable of proceeding with the replication process (as shown by a rapid and sustained decline in the uptake of tritiated thymidine) and ultimately, they die.

Overall, this behaviour of wild type rodent cells is in contrast with the way normal human skin fibroblasts subjected to DNA injury react under equivalent conditions. In human cells recovery of DNA synthetic rate, as measured by cell autoradiography after a dose of $5\text{J}/\text{m}^2$, is initiated between 3 and 6 hours and reaches a maximum value 65-75 % that of unirradiated control by 12 hours after irradiation which is maintained for up to 24 hours without increasing further (Rudé and Friedberg, 1977). This is concomitant with a marked G2 arrest and a prolonged delay of entry into mitosis shown by these

cells after UV (normal human diploid fibroblasts delay entry into mitosis for up to 32 hours if irradiated in S) (Wang and Ellem, 1994). Apparently, this delay is required for the DNA to be repaired before the cell proceeds safely into mitosis. A human cell line deficient in NER would not recover from this arrest and indeed this is in accordance with the detailed biochemical study performed on the human XPA-deficient cells (see previous section).

Both wild type CHO cell lines seem to recover an unirradiated-like rate of DNA synthesis in a very short period of time (8-10 hours after UV) and presumably, this means that they are ready to proceed with the following phase of the cell cycle, mitosis. Given the short time of arrest the DNA-repair process is probably not completed and they proceed on a partially damaged template. We know this because the rate of repair in these cells is very low (Meneghini et al., 1981) and according to the studies described in the previous section (see section 4.3.1), the burden of ESSs that these cells carry at the time that the recovery from arrest is seen (10 hours) is still very high. The UV dose used in the experiment described in section 4.3.1, as has been discussed previously, is lower than that used in the tritiated thymidine incorporation experiments described in sections 4.3.4 and 4.3.5, but it would be logical to assume that the number of unrepaired CPDs in the latter experiments would be even higher, which reinforces the idea that the bypass mechanism is of great importance in rodent cells. The mechanism that these cells use to bypass this arrest is not known.

4.4 Discussion

DNA damage leads to a rapid and dose-dependent inhibition of replication. This damage-induced arrest of DNA synthesis has at least two components. One is the actual blockage of replication by the damage (Edenberg, 1976; Berger and Edenberg, 1986; Moore et al., 1981) and is due to delay in fork progression (Lehmann, 1972; Dahle et al., 1980; Meneghini et al., 1981; Meneghini and de Mello Filho, 1983; Painter, 1985). The other is the blockage of replicon initiation (Doniger, 1978; Kaufmann et al., 1980; Cleaver et al., 1983). Ultraviolet light damage affects mainly DNA-fork progression (Edenberg, 1976; Berger and Edenberg, 1986) although some reduction of replicon initiation can be seen, especially at low doses (Cleaver et al., 1983; Painter, 1985).

From *in vitro* studies it is known that elongation can be blocked or slowed when the polymerase is unable to find an appropriate matching nucleotide for an altered, missing or misaligned base (Moore et al., 1981). On the other hand, the block to replicon initiation appears to affect initiation in an entire replicon cluster (Painter and Young, 1976; Kaufmann et al., 1980). Inhibition of initiation of replication and inability to bypass lesions are finally overcome by unknown mechanisms (Painter, 1985; Griffiths and Ling, 1987; Bierne and Michel, 1994).

In general, after DNA-damage is inflicted, repair takes place and replication is inhibited. If the repair mechanism that deals with the type of damage inflicted is not present, it is possible to think that replication would ensue. This is indeed the case in human cells derived from AT (ataxia telangiectasia) patients (Shiloh et al., 1985). This reflects a deficiency of AT cells in the repair of DNA damage which is coupled to replication (Hanawalt and Painter, 1985).

The relative recovery of DNA synthesis after damage (UV irradiation) also varies depending on the animal species under study. Rodent cells recover DNA replication and progress through the cell cycle much faster than human cells. When hamster cells in S are irradiated with UV, they arrive at mitosis within 15 hours of exposure, with many chromatid type aberrations. By contrast, the entry of human cells is delayed by ~30 hours (Wang and Ellem, 1994). After UV the long-lived DSBs retard the progression of the S-phase human cells through the cycle, and if the DSBs are not repaired (as happens in XPA cells) the cells arrest in G2. Rodent cells either repair or bypass the UV induced lesions, but do not generate DSBs, which enables the cells to reach the first mitosis (Shoshana Squires and Robert Johnson, Cambridge, personal communication). Wild type human cells show significant blockage of replication after exposure to 5 J/m² but not to 2.5 J/m² and this arrest has been shown to last for 5 hours after which replication continues as shown by DNA fibre autoradiography (Griffiths and Ling, 1989). Excision repair deficient human cells show significant blockage both immediately and 5 hours after irradiation even at lower UV doses (2.5 J/m²) (Griffiths and Ling, 1989). In the case of CHO cells (V-79 CHO) dimers have been shown to constitute a block of fork displacement during the first 5 hours after exposure. This chain growth arrest is bypassed within 10 hours after irradiation (Dahle et al., 1980).

Most of the work in this area has been carried out using human and Chinese hamster cells and it was of interest to investigate the behaviour of mouse cells.

In the present work, we have investigated the capacity of DNA-replication arrest of Chinese hamster and mouse cells after UV as well as the capacity of these cells to recover from such arrest and we have looked into the possible role of ERCC1 in this process.

First, it has been possible to confirm that wild type mouse cells (NER proficient) repair replicating DNA preferentially, as shown by their ability to remove DenV-induced SSBs more rapidly from replicating DNA than from the bulk DNA. On the other hand, as expected, ERCC1-defective mouse cells do not repair DNA and the number of UV-inflicted SSBs remain high both in the bulk and replicating DNA. This repair in wild type rodent cells is nevertheless moderate.

Second, it has been shown that the arrest that both wild type and ERCC1-deficient mouse and CHO cell lines show after UV is shorter than the arrest shown by a human cell line that cannot repair its DNA (XPA). This has also been reported before (Meneghini et al., 1981). In both mouse cell lines (PF20 and PF24), nearly all the DenV-associated DSBs disappear by 3 hours. This indicates that after a period of stalling, the DNA-polymerases start to bypass the pyrimidine dimers without leaving a gap in the daughter strand, and/or that the blocking pyrimidine dimers have been repaired (in the case of PF20 cells only) before replication is resumed. In the case of CHO cells, however, the period of stalling is longer (about 6 hours). Overall, there does not seem to be a requirement for functional NER in order for rodent cells to bypass fork progression arrest due to UV damage as shown by the fact that, in both the mouse and the CHO cells, both the NER-proficient and the NER-deficient cell strains show the same behaviour.

Third, there was an indication that ERCC1-deficient cells arrest fork-progression for even shorter times than their wild type counterparts. In order to investigate this latter point, experiments were designed that allowed us to look into this particular aspect in more detail. Using this technique it was possible to determine the cell cycle response of rodent cells to UV irradiation. There was no indication that ERCC1 null mouse

cells behave differently to the wild type cells in terms of arrest of [^3H]Thymidine incorporation in the first hours after UV damage. By 4-6 hours all the wild type cell types studied start recovering from the reduction in [^3H]Thymidine incorporation. By 10 hours the levels of incorporation in CHO wild type cells go back to pre-UV levels, indicating that the cells have recovered and can progress through the cell cycle. This recovery of DNA synthesis has been reported before in normal human and Chinese hamster cells for low UV fluences (Rudé and Friedberg, 1977; Dahle et al., 1980; Ventura and Meneghini, 1984). No recovery was seen in ERCC1 null cells, which is in accordance with the fact that these cells cannot repair UV-induced DNA damage and therefore are highly sensitive to UV-induced cell killing. Experiments with unsynchronised CHO9 and CHO 43-3B cells have been published that support the data presented here in terms of the kinetics of recovery from UV-induced DNA synthesis inhibition (Wood et al., 1982). In their study, Wood *et al.* also show that at low UV doses (0.5 J/m^2) CHO9 and CHO 43-3B cells show the same response to UV irradiation in terms of DNA synthesis inhibition. At higher UV doses, CHO 43-3B cells show an impairment in their recovery from UV as shown by their kinetics of recovery from DNA synthesis after higher irradiation fluences (1.0 and 2.5 J/m^2) (Wood et al., 1982) in a manner equivalent to that reported here with the 20 J/m^2 dose. A failure to recover DNA synthesis after UV irradiation (5 J/m^2) has been shown to occur with XPA cells (Rudé and Friedberg, 1977). However, at lower UV fluences (1 J/m^2), XP cells can bypass the replication arrest (Kaufmann and Cleaver, 1981).

When the response seen in our thymidine incorporation experiments is correlated with the results obtained from the previous set of experiments, some facts to be taken into account. In the case of the NER-proficient cells it is clear that the data obtained from the AFIGE analysis and the data obtained from the tritiated thymidine incorporation experiments can be easily reconciled. Fork progression (as determined by the AFIGE procedure) is restored in the wild type cells approximately 3-6 hours after UV irradiation (10 J/m^2). This has already been shown for V-79 CHO cells which only show arrest of fork progression during the first 5 hours after UV irradiation (Dahle et al., 1980). DNA synthesis (as measured by tritiated thymidine incorporation levels) reaches unirradiated control levels 10 hours after UV (20 J/m^2) in the same cells. Dahle et al. also report total recovery of chain growth by 10 hours after UV exposure.

One explanation for the lagged response in terms of the recovery of DNA synthesis levels could be that since the latter experiments were performed under more damaging conditions, the cells need more time to bypass the arrest. Another explanation is that although fork progression is fully restored earlier, DNA synthesis takes a bit longer to go up to normal levels, because the synthesis of DNA that takes place only starts after the fork movement is restored. It is interesting to note that DNA synthesis levels start to go up precisely at 4 hours post-irradiation, when fork progression is fully restored.

In the case of the NER-deficient cells (both mouse and CHO) the two experimental procedures seem to give contradictory results. Although both CHO 43-3B and PF24 cell strains seem to behave in an equivalent manner to their respective parental cell lines in terms of fork progression arrest as shown by the AFIGE technique, they present a different response in terms of DNA synthesis rate recovery. These NER-deficient cell lines bypass fork progression arrest, but they show a permanent arrest of DNA synthesis. Wood et al. also report no recovery in DNA synthesis in CHO 43-3B cells after 1 and 2.5 J/m² (Wood et al., 1982), which supports the results obtained with the [³H]thymidine uptake procedure. One explanation for this discrepancy in the results could be the difference in the UV fluences used for both experimental procedures. For the fork progression assessment experiments, the UV dose used was lower than that used for the DNA synthesis rate recovery experiments. In the former case, it is possible that the cells, even though they cannot repair their DNA, manage to escape the arrest somehow, through a yet unidentified bypass mechanism. When the UV dose is higher and the damage inflicted increases, the bypass mechanism is not enough anymore and NER is then required in order to achieve recovery from arrest. The other possibility is that after low UV fluences, NER-deficient cells still possess a residual capacity for repair of UV-induced lesions that is overwhelmed at higher UV doses (higher number of lesions) (Wood et al., 1982). Under these circumstances, and if NER is absent, further progression into the following phase of the cell cycle is prevented and the arrest is therefore permanent. This checkpoint probably takes place in G2 and prevents the occurrence of mitosis in the presence of damaged DNA. Another possible explanation for the conflicting results is that, given the complexity of the first experimental procedure, the interpretation of the data obtained by this method is dependent on too many parameters (such as DNA conformation and migration patterns

on the gel, distribution of UV-induced lesions along the bulk or replicating DNA, very complicated mathematical calculations) and it is difficult to interpret straightforwardly. This would make the results obtained in this way unreliable.

ERCC1 does not seem to play a significant role in the process of replication arrest as indicated by the fact that CHO cells defective in ERCC2 (UV5) behave in the same way as cells defective in ERCC1 (43-3B). From this study it can only be said that in the presence of UV-damage, the absence of a proficient DNA repair mechanism in rodent cells does not seem to be a stringent factor in the progression of fork movement. However, it seems that NER-deficient cells manage to halt cell cycle progression and ultimately they prevent gross abnormalities from being passed on to the daughter cells. However, this does not explain why ERCC1-deficient cells (and in particular hepatic cells) and not ERCC2-deficient cells show such an aberrant phenotype. A plausible explanation for this difference comes from studies performed by Melton *et al.* (Melton et al., 1998) that show that ERCC1 has a unique, albeit indirect, role in the mechanisms that determine progression into the next phase of the cell cycle. This is not new since ERCC1 has been implicated in recombination and other eukaryotic ERCC1-homologues have been shown to be involved in an array of different functions from mating-type switch in yeast to meiotic recombination in *D. melanogaster* (see Introduction, section 1.1 and Conclusion).

As has been shown before, in the presence of UV damage, rodent cells seem to bypass a key checkpoint (S-checkpoint) and proceed through replication despite the fact that repair is incomplete, and the gaps created after UV are still present and represent a serious risk for the rodent DNA integrity (see Introduction, section 1.4). This is also the case for rodent cells defective in NER, i.e., ERCC2-deficient cells. In fact, rodent cells possess a mechanism that ensures that the DNA integrity is somehow restored so that it is possible to go through replication in the absence of 'properly'-repaired DNA. Such a system is called 'illegitimate recombination'.

Studies carried out by Melton and co-workers suggest that cells that lack ERCC1 are deficient in 'illegitimate recombination' and are therefore more prone to carry through the UV-induced gaps in their DNA into the next phase of the cell cycle (Melton et al., 1998). Although the resulting product is DNA that has breaks in it, the cell is allowed

to proceed through replication and damage is carried through to the G2 phase of the cell cycle where the strong arrest occurs. This would result in cells going through normal, non-stringent rounds of replication in the absence of cell division, which would explain the observed polyploidy and aneuploidy in the ERCC1-deficient hepatocytes. In the case of ERCC2-deficient cells the DNA would be recombined, and the progression through the cellular cycle would continue normally with normal rounds of mitosis and no aberrant nuclear phenotype.

In summary, rodent cells, as opposed to human cells, accept unrepaired DNA damage, and are subsequently exposed to high rates of mutations. That they tolerate this is probably due to the fact that their generation time and their life span are short which allows them to reproduce successfully before the onset of lethal cancers and tumours. When the rough patch up mechanism they have evolved to quickly overcome gross DNA aberrations is not present, as in ERCC1-deficient mice, they do not even reach maturity.

It is a fact that rodents show a very high incidence of cancers and tumours with increasing age. A balance appears to exist between the necessity to repair DNA-damage in order to be able to successfully pass on the genetic pool to the next generation and the time, energy and resources that it takes to do so. In humans, who have longer life spans than rodents and lower reproductive success, it is therefore necessary to maintain a strict control of mutations and precise, stringent and tightly regulated cellular responses are a necessary 'investment'.

Chapter 5

In Vitro Studies: Effect of UV on the p53 and p21 Responses in ERCC1–Deficient Primary Fibroblasts

5.1 Introduction

Human patients suffering from XP, a syndrome caused by defects in NER, show an increased incidence of skin cancer in sun-exposed skin areas. Consequently, there have been extensive studies on the response of cancer-related proteins, such as p53, to the effects of UV exposure in cells derived from these patients. As expected, studies of human cell lines defective in NER genes, such as XPD, show that defects in this DNA repair pathway make these primary fibroblasts more sensitive to the effect of UV light in terms of the p53 response, i.e. lower UV doses are needed to elicit a significant p53 response in these cells (Dumaz et al., 1998).

ERCC1 is essential for the NER process. The phenotype of the mice lacking ERCC1 has no resemblance to any known human disease related to a defect in this DNA-repair mechanism. The fact that these transgenic mice die so young makes the *in vivo* study of their response to UV impossible. Despite the interest in the study of the relationship between DNA damage, DNA repair, and cell cycle control, there are few published studies of p53 responses to UV damage in ERCC1-deficient cells (Melton et al., 1998).

This chapter presents a study of the response of both p53 and p21 to UV in primary fibroblasts from the already mentioned genotypes: wild type, p53+/+ERCC1-/-, p53-/-ERCC1+/+ and p53-/-ERCC1-/-. The culture of primary mouse fibroblasts presented some limitation. Growth of these cells required densities to be at least 70-80% confluence in order to maintain the cultures under proliferating conditions and even very slight reductions in these densities led the cells to arrest of cell growth and death. Another characteristic of these cultures is their rapid senescence. Since the studies that were going to be performed on them involved responses in terms of the induction of genes that have also been related to death-related pathways, it was important to carry out these experiments soon after their isolation from the embryos had taken place. All primary fibroblasts used in this study were used at a low passage number (passage 2 when the density of cells allowed work at this stage and passage 3 when the initial density of cells was low).

5.2 Results

5.2.1 Low UV doses are sufficient to elicit a p53 response in ERCC1-deficient primary fibroblasts.

The kinetics of the p53 response to different UV doses was assessed in wild type and ERCC1-/- primary fibroblasts. Cells were irradiated with different UV doses (5, 20 and 50 J/m²) and immunoblotting was carried out to show stabilisation of p53 at different time points after the UV irradiation (for details see Materials and Methods, section 2.2.11, subsection 2.2.11).

Mouse wild type primary fibroblasts show no accumulation of p53 at low levels of UV irradiation (5 J/m²) (see Fig.5.1 A). This is in accordance with studies carried out by Dumaz *et al.* in normal human and XPD primary fibroblasts (Dumaz *et al.*, 1998). Dumaz *et al.* also report that XPD deficient cells are more sensitive to UV than their wild type counterparts in that, under the same low UV dose (5 J/m²), they induce p53 readily whereas the wild type human cells show no detectable response. This is also seen in the ERCC1 null primary fibroblasts (see Fig.5.1 A). The stabilisation of p53 after 5 J/m² is detectable 2 hours after UV irradiation and is maintained for at least 30 hours. This long p53 accumulation is also seen in XPD fibroblasts, described by

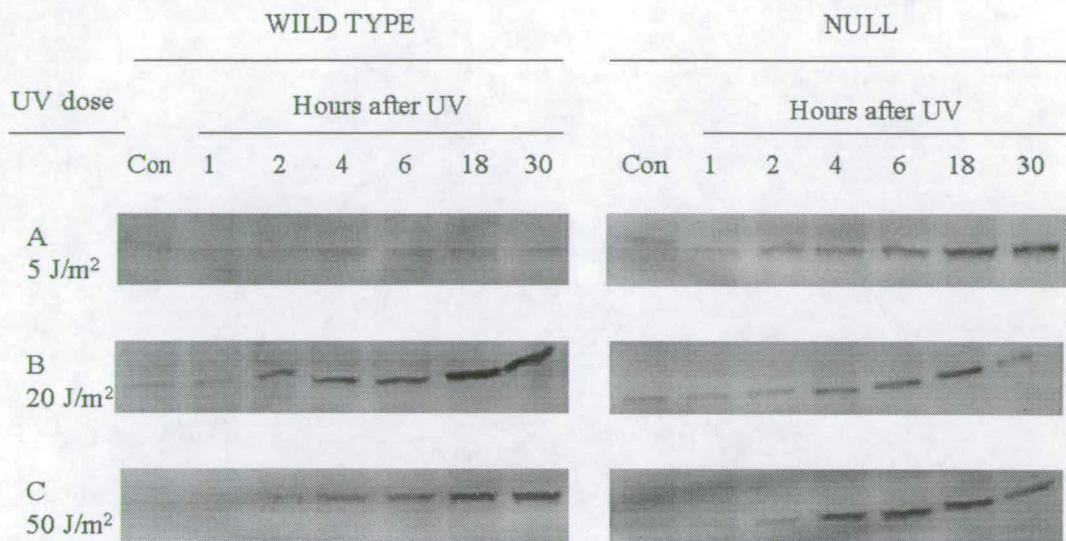


Figure 5.1: Stabilisation kinetics of p53 after varying doses of UV in wild type (left hand panels) and ERCC1-/- (right hand panels) mouse primary fibroblasts. A) 5 J/m²; B) 20 J/m²; C) 50 J/m². The level of p53 protein was determined in unirradiated controls (Con), and at different intervals after UV (1, 2, 4, 6, 18 and 30 hours).

Dumaz and co-workers, which show sustained p53 stabilisation up to 120 hours after low doses of UV irradiation.

When mouse primary fibroblasts are challenged with a dose of 20 or 50 J/m² (see Fig.5.1 B and C), both the wild type and the ERCC1-deficient cells behave in the same way and follow the same kinetics. They accumulate p53 from as soon as 2 hours post-irradiation and the response is maintained for at least 30 hours. This mouse wild type response is in contrast with the one described for the human wild type cells as reported by Dumaz *et al.* Normal human fibroblasts show a decrease in the amount of p53 protein back to normal levels 24 hours post-irradiation (20 J/m²). This may reflect a difference in the way human and mouse fibroblasts deal with DNA damage. Human cells tend to completely repair their DNA before proceeding with their cell cycle, whereas rodent cells can progress through theirs with damaged DNA, which would result in more prolonged p53 responses.

In general, the kinetics of induction of p53 under these conditions are also supported by the findings of Lu and Lane (Lu and Lane, 1993). In their work, Lu and Lane report

that after a dose of either 10 or 50 J/m², mouse prostate cells show stabilisation of p53 after a lag time of 2 hours and its levels remain high for at least 30 hours.

We do not see a difference in the basal unirradiated levels of p53 amongst the different genotypes studied here. In both wild type and ERCC1-deficient there were barely detectable levels of p53 in unirradiated fibroblasts and in no case was an increase seen in the ERCC1 deficient cells. p53 has been implicated in the transcriptional activation of p21 on many occasions (El-Deiry et al., 1993; El-Deiry et al., 1994; Cox, 1997; Isaacs et al., 1997; Shigemasa et al., 1997; Takahashi and Suzuki, 1994; Waldman et al., 1995), but p21 has also been shown to be transcriptionally activated in a manner independent of p53 (Cox, 1997; Janson et al., 1997; Johnson et al., 1994; Michieli et al., 1994). In order to assess the situation regarding the relationship between p53 and p21 in the mouse primary fibroblasts in response to UV irradiation we performed a number of experiments to study the induction of p21 under different UV doses in wild type, ERCC1-deficient and also p53-deficient and p53 and ERCC1 double null mouse primary fibroblasts.

5.2.2 Basal levels of p21 are elevated in ERCC1-deficient primary fibroblasts.

As shown in Fig. 5.2 the basal levels of p21 in ERCC1-deficient primary fibroblasts is higher than that of the wild type cells (as has already been shown in liver). Therefore, p21 seems to be induced in these NER-deficient cells in the absence of exogenous DNA damage and in the absence of a detectable p53 stabilisation (see previous section). These permanently-induced basal levels of p21 may have consequences in the response of these cells to UV. In fact, we fail to see proper induction of p21 in these primary fibroblasts (see below).

It is noteworthy that in the case of primary fibroblasts deficient in p53, both p53 -/- ERCC1+/+ and double nulls, the basal levels of p21 are reduced to a tenth of those seen in the wild type cells. This observation is in contrast with the data obtained from the livers (Fig. 3.14, Chapter 3; for a summary see Table 5.1).

It seems likely that p21 transcription in the absence of exogenous damage, as indicated by basal levels of p21 *in vivo* and *in vitro* have different p53 requirements. In hep-

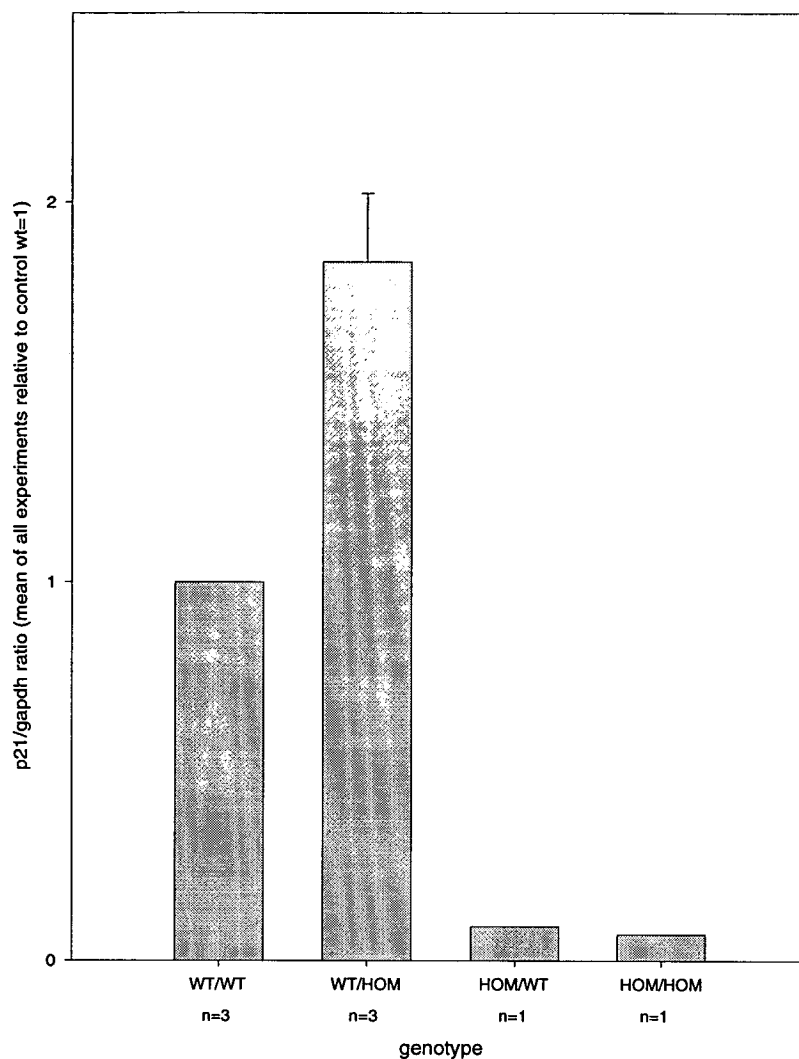


Figure 5.2: p21 basal levels in primary fibroblasts corresponding to the four different genotypes. The bars show the means of the p21/GAPDH ratio for all the samples of a given genotype. An n value of 1 represents 2 determinations on independent dishes from a single embryonic fibroblast culture. Standard errors are shown.

atocytes *in vivo*, transcription of p21 is p53-independent and in primary embryonic fibroblasts *in vitro* it is dependent on p53. This also supports previous *in vitro* findings (Reinke and Lozano, 1997) that p21 expression is dependent on p53 and reinforces the idea that, *in vitro*, p21 expression is p53 dependent and, *in vivo*, p21 regulation is p53 independent, at least under the conditions observed here.

<i>Genotype</i>	<i>Primary Fibroblasts</i>	<i>Liver</i>
p53+/+ERCC1+/+	1	1
p53+/+ERCC1-/-	2	2
p53-/-ERCC1+/+	0.1	1
p53-/-ERCC1-/-	0.1	2

Table 5.1: Summary table of p21 basal transcript levels in primary fibroblasts and liver tissues. p21 levels are expressed as p21/GAPDH ratios.

5.2.3 p21 induction is impaired after high UV doses in ERCC1-deficient fibroblasts

In light of the previous results, we were interested to see the response of the four different types of fibroblasts to UV damage. The induction kinetics of p21 after different doses of UV irradiation in each one of the primary fibroblast classes are shown in Fig. 5.3.

The p21 response after low UV doses, 5 J/m², (Fig. 5.3, 5 J/m²) is similar for all the genotypes studied although there is an indication of a higher p21 induction in p53hom/ERCC1hom primary fibroblasts at the longer time points (7 and 24 hours after UV). Briefly, p21 message levels are induced around two-fold three hours after UV and the induction is still maintained 7 hours after irradiation. In the wild type primary fibroblasts p21 transcript levels had gone back to control, unirradiated levels, whereas in the other three groups of cells it remained high. Reinke *et al.* (Reinke and Lozano, 1997) report a 10-fold induction of p21 transcript in rat embryo fibroblasts 6 hours after a 10 J/m² dose of UV which persists for at least 48 hours. We do not see such a prolonged induction of p21 after 5 J/m² but we see sustained induction after 20 J/m² (see below). The UV dose used by Reinke *et al.* is intermediate between our two UV doses and possibly reflects the minimal amount of UV damage required for a prolonged p21 induction.

There is no evidence from this study that ERCC1-deficient cells are defective in the p21 induction after low UV irradiation or that their p21 induction is more sensitive to UV than that of wild type or p53 deficient cells. Reinke and co-workers also report that this p21 induction is dependent on p53-binding sites being present in the p21 promoter, suggesting that p21 is activated via a p53-dependent mechanism. Their experiments

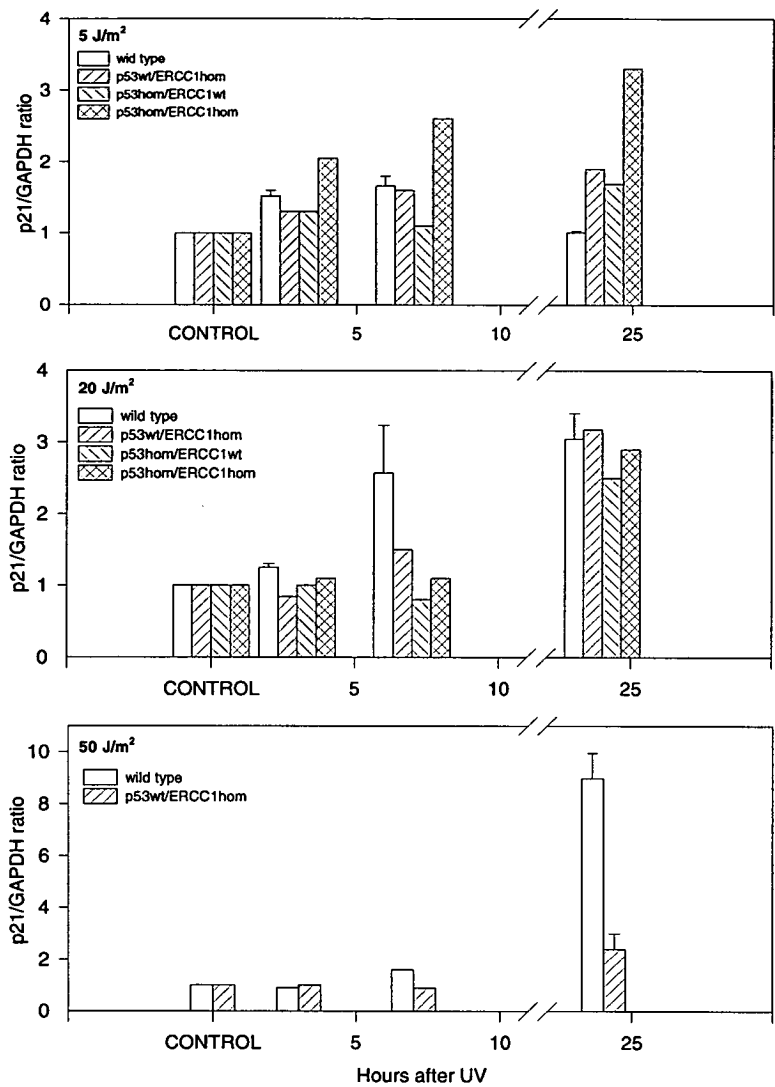


Figure 5.3: Induction of p21 in asynchronous mouse primary fibroblasts (wild type, ERCC1 null, p53 null and double null) collected at different time points (3,7 and 24 hours) after varying doses of UV. All values refer to p21/GAPDH ratios within the same genotype. Standard errors are shown when possible.

were based on an *in vitro* assay using a construct containing a p21 promoter and the reporter gene luciferase. Here we present *in vitro* evidence that suggests that p53 is not required for the transcriptional activation of p21 after UV damage, as shown by detectable p21 induction in double null primary fibroblasts 7 and 24 hours after UV irradiation. The discrepancy in these results may be due to differences in the responses to UV in mouse and rat primary fibroblasts or to the differences in the experimental approach.

When the UV dose of 20 J/m² is considered, it becomes more evident that NER-deficient cells are impaired in their p21 response (Fig. 5.3, 20 J/m²). Wild type cells noticeably induce p21 levels seven hours after UV (3 times unirradiated control levels) and this induction is maintained for at least 24 hours. At 7 hours post-irradiation none of the mutant cell lines show a clear increase in p21 transcript levels. However, 24 hours after irradiation all four cell types seem to reach similar levels of induction. High levels of p21 protein have been reported after a UV dose of 20 J/m² in TTD/XPD human fibroblasts 24 hours after irradiation, which is consistent with these data (Dumaz et al., 1998). This may reflect a delay in the response to UV damage in terms of p21 induction in cells lacking ERCC1. It is also noteworthy that both primary fibroblast cell lines lacking p53 show p21 induction 24 hours post-irradiation, although this has to be put in the context of low overall levels of p21 transcript in these cells (1/10 of that seen in wild type and ERCC1-/- primary fibroblasts) (see Fig. 5.2).

The results for the highest UV dose (50 J/m²) are shown in Fig. 5.3, 50 J/m². For these experiments only wild type and ERCC1 -/- cells were considered. Wild type cells fail to induce p21 transcript levels at 3 and 7 hours post-irradiation. However, the induction in p21 message reaches a ten-fold increase 24 hours after UV damage. Similar results have been reported for human normal fibroblasts and rat embryo fibroblasts (Poon and Hunter, 1998; Reinke and Lozano, 1997). In their work, Poon and Hunter describe absence of induction of p21 protein levels 12 hours after high UV doses (60 J/m²) despite apparent accumulation of p53 protein. At these high UV doses, they detected a faster mobility form of p21 (p21 Δ) which is mainly localised to the cytoplasm of the cell and which they propose may have a role in the deregulation of the cell cycle. However, they did not look at later time points and therefore the duration of the absence of p21 induction they see cannot be determined. Due to the lack of appropriate anti-mouse p21 antibodies it was not possible for us to look at the protein expression of p21 in this work, but it would be interesting to see if p21 Δ is also expressed in these mouse fibroblasts. Reinke and Lozano also note the absence of p21 message induction after high doses of UV (50 J/m²). They propose that the accumulation of damage after this UV dose is so great that it interferes with transcription and ultimately leads to apoptosis.

The fact that ERCC1-deficient primary fibroblasts fail to induce p21 24 hours after high UV doses (50 J/m²) seems to support this theory. After high UV doses, in the absence of a proficient NER mechanism, damage accumulates and remains unrepaired, which could explain the long-term inhibition of the p21-induction seen in these cells. In the case of wild type cells which can repair the damage it is possible that after a transient inhibition of transcription, the cells finally manage to overcome the damage and the cellular machinery recovers the ability to keep functioning.

5.3 Discussion

There is extensive evidence on the relationship between DNA damage, cancer-related genes such as p53 and p21 and cell-cycle regulators in different cell types both of rodent and human origin (Gadbois and Lehnert, 1997; Khan et al., 1997; Kuwano et al., 1997; Merlo et al., 1997; Rathmell et al., 1997; Smith et al., 1994; Smith et al., 1995; Smith and Fornace, 1996). These studies suggest that p53 and p21 are regulated differently according to both the cell type (Gadbois and Lehnert, 1997) and the type of agent that causes that damage (Janson et al., 1997). For example, p53 can be accumulated in cells without p21 being transcriptionally activated (Khan et al., 1997). Moreover, although p21 message has been shown to be induced by p53 (El-Deiry et al., 1993; El-Deiry et al., 1994; Cox, 1997; Isaacs et al., 1997; Shigemasa et al., 1997; Takahashi and Suzuki, 1994; Waldman et al., 1995), it has also been shown to be induced via p53-independent pathways (Cox, 1997; Janson et al., 1997; Johnson et al., 1994; Michieli et al., 1994). The end result of these signalling and control pathways, that is, the response of the cells to the exogenous DNA damage also varies depending on the cell type. Either apoptosis (Merlo et al., 1997; Reinke and Lozano, 1997) or growth arrest (Smith and Fornace, 1996; Reinke and Lozano, 1997) have been reported as the end result of the p53-mediated response.

A direct relationship between p53 and DNA repair has been proposed by Smith *et al.* (Smith et al., 1995). The authors present evidence from human colon carcinoma RKO cells, in which p53 function is abrogated, for reduced repair of UV-induced DNA damage. p21 has also been linked directly to DNA repair (Li et al., 1996; McDonald et al., 1996). Li and collaborators demonstrate by *in situ* experiments (quantitative

immunoblotting) on human W138 diploid fibroblasts, that the DNA repair capacity of the cells has a bearing on the expression of both PCNA and p21 proteins in the nucleus of these cells. Furthermore, they suggest that both PCNA and p21 have a role in linking DNA replication, DNA repair and cell cycle progression. McDonald *et al.* report that p21 function is required for DNA repair of a reporter plasmid that had been irradiated with UV and introduced in colon cancer cells. p21^{-/-} cells show decreased reporter activity following transfection when compared to p21^{+/+} cells.

Overall, the dynamics of the interaction between DNA damage, its recognition pathways and the mechanisms involved in the cellular response to the injury (cell cycle regulation) are complex and generalisation is not easy. Literature is extensive in this area and the response of a number of different cell types to UV damage in terms of p53 and p21 activation has been studied in detail (see Table 5.2). In summary, all the cell types studied in the above experiments (see Table 5.2) respond to UV by inducing p53 and p21 with the end result being cell cycle arrest, and in the case of DNA repair proficient cell lines, their DNA being repaired if the damage is not extreme. When too much DNA damage occurs, the cell usually decides to ‘commit suicide’ (apoptose). In this case UV has been proposed to interfere with the transcription machinery (Reinke and Lozano, 1997), and although post-translational stabilisation of p53 occurs even after the highest UV doses (50 & 60 J/m²) (Reinke and Lozano, 1997; Poon and Hunter, 1998; Lu and Lane, 1993), p21 mRNA (Reinke and Lozano, 1997) and protein (Li *et al.*, 1996; Poon and Hunter, 1998) are not induced.

In general, the NER-defective cell lines are more sensitive to UV (they stabilise p53 at very low UV doses: 2.5 J/m²) (Dumaz *et al.*, 1998) but their response to higher UV doses in terms of the kinetics of the accumulation of p53 are similar to those seen in their normal counterparts (Lu and Lane, 1993). Apparently, the half-life of the p53 protein is elevated in non-irradiated XPA, XPB and TTD human fibroblasts (Abrahams *et al.*, 1995). It is also interesting that two cell lines from Bloom’s syndrome patients show no p53 response as reported by Lu and Lane. They also report that the p53 response is delayed in AT cell lines.

All the studies that are presented in Table 5.2 which investigate the relationship between the p53 and the p21 response (Reinke and Lozano, 1997; Poon and Hunter,

<i>Cell Type</i>	<i>Challenge</i>	<i>Response</i>	<i>Reference</i>
NHDSF ¹	UV (10J/m ²)	p53 stabilisation (Biphasic). Noticeable after 1h. Sustained for up to 48 h.	(Abrahams et al., 1995)
XPA ² , XPB ² , TTD ³	UV (10 J/m ²)	p53 stabilisation (Monophasic). Constitutive increase in p53 half-life.	(Abrahams et al., 1995)
REF ⁴	UV (10 and 50 J/m ²)	p53 stabilisation. Detected after 6h. Sustained for up to 24 h. Dose dependent.	(Reinke and Lozano, 1997)
REF	UV (10 and 50 J/m ²)	p21 mRNA induction. Noticeable after 6h. Sustained for up to 24 h. Not detected after 50 J/m ² .	(Reinke and Lozano, 1997)
NHDF ⁵	UV (30 and 60 J/m ²)	p53 stabilisation. Noticeable at 12 h.	(Poon and Hunter, 1998)
NHDF, S3T3 MF ⁶ , RF ⁷	UV (30 and 60 J/m ²)	p21 protein induction. Noticeable at 12 h. Not detected after 60 J/m ² .	(Poon and Hunter, 1998)
NHDF	UV (12 J/m ²)	p21 protein stabilisation. Noticeable after 3h. Sustained for up to 24 h.	(Li et al., 1996)
NHDF	UV (20 J/m ²)	p53 stabilisation. Noticeable at 3 h.	(Nelson and Kastan, 1994)
XPA	UV (20J/m ²)	p53 stabilisation. Noticeable at 3 h.	(Nelson and Kastan, 1994)
MPC ⁸	UV (10 and 50 J/m ²)	p53 stabilisation. Noticeable after 2 h. Plateau at 12 h. Sustained for up to 30 h.	(Lu and Lane, 1993)
XPA	UV (50 J/m ²)	p53 stabilisation. Noticeable after 2 h. Plateau at 12 h. Sustained for up to 30 h.	(Lu and Lane, 1993)
BS ⁹	UV (50 J/m ²)	No p53 stabilisation in 2 out of 11 BS cell lines.	(Lu and Lane, 1993)
AT ¹⁰	UV (50 J/m ²)	p53 stabilisation. Appears at 3h. Increases significantly at 18 h. Similar to MRC5 NHDF.	(Lu and Lane, 1993)
NHDF	UV (various UV doses)	p53 stabilisation only occurs at the higher UV doses (10 and 20 J/m ²). Reaches a maximum at 24 h. and declines after that.	(Dumaz et al., 1998)
XPD, TTD/XPD	UV (various UV doses)	p53 stabilisation occurs at the lowest UV doses (2.5 J/m ²). At 5 J/m ² appears at 4h. Sustained for 120 h.	(Dumaz et al., 1998)
B3T3 ¹¹	UV (various UV doses)	p53 stabilisation starts at 5 J/m ² . Maximum stimulation at 10 J/m ² . Less stimulation at 50 J/m ² . Appears at 0h. Sustained for 6 h.	(Maltzman and Czyzyk, 1984)

Table 5.2: Some examples of p53 and p21 responses to UV irradiation in various cell types. ¹NHDSF: Normal Human Diploid Skin Fibroblasts; ²XP: Xeroderma Pigmentosum; ³TTD: trichothiodystrophy; ⁴REF: Rat Embryonic Fibroblasts; ⁵NHDF: Normal Human Diploid Fibroblasts; ⁶S3T3 MF: Swiss 3T3 Mouse Fibroblasts; ⁷RF: Rat Fibroblasts; ⁸MPC: Mouse Prostate Cells; ⁹BS: Bloom's Syndrome; ¹⁰AT: Ataxia Telangiectasia; ¹¹B3T3: BALB/c 3T3 nontransformed mouse cells.

1998; Dumaz et al., 1998) point towards a direct role for p53 in the transcriptional activation of p21. This is also supported by other studies with other DNA damaging agents in which abrogation of p53 in NHDF, through expression of a dominant negative p53 mutant, results in the lack of induction of p21 after bleomycin-induced DNA damage (Wyllie et al., 1996). However, a p53-independent p21 response has been reported after treatment of p53-deficient embryonic mouse fibroblasts with butyrate (Janson et al., 1997). This may reflect differences in the behaviour of different cell lines to different DNA-damaging agents. We wanted to investigate this aspect of the relationship between p53 and p21 in the context of DNA repair and in order to do so we looked at the stabilisation kinetics of p53 in normal and ERCC1-deficient mouse primary fibroblasts and also we have been able to look at the induction of p21 in these cells and in mouse cells defective in p53 and in cells lacking both p53 and ERCC1.

p53 is not induced under low UV doses (5 J/m^2) in wild type mouse primary fibroblasts as seen in the studies carried out in our laboratory (see Fig. 5.1 A). These results are supported by studies carried out using human primary fibroblasts (Dumaz et al., 1998). However, in contrast with observations by the same authors we do not see a reduction of p53 protein back to background levels in the mouse cells. In fact, at higher UV doses (20 and 50 J/m^2) p53 stabilisation in mouse primary fibroblasts is seen as soon as 2 hours after irradiation and is maintained for as long as 30 hours (see Fig. 5.1 B and C). The difference in the behaviour of the two cell types can be explained in view of the fact that human cells repair the DNA damage from their genome within 24 hours (Abrahams et al., 1995) and therefore p53 levels go down as DNA damage disappears. This is not the case for rodent cells which are capable of proceeding through their cell cycle with unrepaired DNA (they possess a high tolerance for the presence of CPDs in the non-transcriptionally active parts of their genome) (Orren et al., 1995). Orren *et al.* report that although CHO cells arrest the cell cycle progression in response to UV damage, cycling is not prevented.

There are reports of p53 stabilisation after 5 J/m^2 in B3T3 cells (Maltzman and Czyzyk, 1984). We have constantly failed to see such an induction in wild type mouse primary fibroblasts after the same low UV-dose, but this difference may be due to the different origin of the cell lines used in these two studies. B3T3 cells are a non-

transformed immortalised cell line and the cells reported here are primary embryonic fibroblasts, which could explain the different p53-stabilisation kinetics seen in the two cell lines. As can be seen below, our results are in accordance with the studies carried out by Lu and Lane (Lu and Lane, 1993), who used a similar experimental approach to ours for the detection of p53.

ERCC1-/- cells, on the other hand, accumulate p53 after 5 J/m² (see Fig. 5.1 A). This is in accordance with observations by Dumaz and co-workers (Dumaz et al., 1998) in human cells deficient in XPD. However, when ERCC1-deficient cells are irradiated with the higher UV doses, no difference between their behaviour and that of the wild type cells is seen.

We do not see a delay or a defect in the induction of p53 as reported by Lu and Lane for BS cells (2 out of 11 primary fibroblast cultures derived from patients suffering from BS are reported to fail to accumulate p53 after UV irradiation, even 18 hours after exposure). Lu and Lane's report on the absence of p53 accumulation after UV irradiation in 2 BS primary fibroblast cell lines is the only one out of the studies discussed here that shows that a defect in the DNA damage repair pathway can lead to complete inactivation of the p53 response after UV irradiation. As will be seen in future chapters we have detected a similar defect in a cell line belonging to a patient with a premature ageing syndrome. The implications of this phenotype will be discussed in Chapter 6.

The four genotypic classes of cells used in this experiment allowed us to look for the role of p53 in the expression of p21. We had seen before in liver tissue that the basal levels of p21 were elevated in ERCC1-/- livers even when p53 was not present. Also there was no reduction on the basal levels of p21 in DNA-repair proficient p53 null cells (see Table 5.1). We concluded that p21 expression *in vivo* in mouse liver is p53 independent. This is in contrast with the results obtained with unirradiated cultured primary embryonic fibroblasts derived from the same animals. Under these conditions, p21 levels decrease 10-fold in p53-/- cells when compared to wild type levels. Thus, we conclude that under culture conditions primary fibroblasts exhibit a p53-dependent expression of p21. Although the overall levels of p21 message in these p53-deficient cells were very low we could still detect some induction of p21 transcript 24 hours after

UV irradiation (3 fold induction in p53 null primary fibroblasts after 20 J/m²; 3 fold induction in double null primary fibroblasts after 5 J/m² and 20 J/m²).

The induction of p21 message has also been looked at in these mouse primary fibroblasts. In wild type cells irradiated with the lowest UV dose, p21 is induced 2-fold after UV as detected at 3 and 7 hours post-irradiation and its levels go back to normal after 24 hours. This is in contrast with the lack of detectable induction of p53 at any time in these cells after the same UV dose (Fig. 5.3, 5 J/m²). This result argues against a UV-induced p53-dependent p21 response in these cells as opposed to the situation in unirradiated fibroblasts. This dose resulted in a similar induction of p21 in all the different genotypic classes. There is also an indication of p21 being approximately 3 fold elevated 24 hours after UV irradiation in cells lacking both p53 and ERCC1, although, given the fact that the experiments were only performed once, this could be due to variability in the measurements.

With the dose of 20 J/m², p21 was induced up to 3 fold in the wild type cells 7 hours after UV challenge, its levels remaining high throughout the experiment. This response is in accordance with the pattern of p53 expression seen in these cells irradiated with the same dose of UV and indicates a dose-dependent induction of p21. In the ERCC1-deficient cells p21 was not induced after this UV dose in a significant manner (Fig. 5.3, 20 J/m²) up until the last time point at which p21 mRNA levels were equivalent to those seen in the wild type cell. This is in contrast with the normal p53 response seen in these cells after this UV dose. This fact argues again against a UV-induced p53-dependent p21 induction.

Bearing in mind that the basal levels of p21 are dramatically reduced in the p53^{-/-} and the double null fibroblasts, it is noteworthy that we can detect a 3-4 fold increase in p21 transcript in these two types of cells over unirradiated control levels, 24 hours after UV irradiation, equivalent to that seen in p53^{+/+} cells. This points towards a mechanism of p21 induction which is p53 independent at the later time points. Moreover, the fact that the levels of p21 induction in p53^{+/+} ERCC1^{-/-} cells seems to be equivalent to that of the p53^{-/-} cells points towards an overall redundancy of p53 in the UV-induced transcriptional activation of p21 in this system.

Under the highest UV doses, there was a clear difference in the way wild type and ERCC1-/- cells behaved. Wild type cells failed to induce p21 at the first two time points (3 and 7 hours after UV) but the levels of p21 message had increased up to 10 fold 24 hours after irradiation. On the other hand, ERCC1-deficient cells showed an impaired induction of p21 even 24 hours post-irradiation when compared to their wild type counterparts. Absence of p21 induction after high UV doses has been reported before (Poon and Hunter, 1998; Reinke and Lozano, 1997). In the case of the studies carried out by Poon and collaborators, normal human diploid fibroblasts and rat fibroblasts show no induction of p21 at 12 hours post-irradiation (60 J/m^2). However, they present no data for later time points. Reinke *et al.* looked at the expression of p21 up to 48 hours after UV challenge (50 J/m^2) and could not detect induction of p21 at that time. This is in contrast with our findings that p21 is in fact highly induced 24 hours after UV-irradiation in wild type primary embryonic fibroblasts, but coincides with the p21 response we see in the ERCC1-deficient cells. They also report very high induction of p21 in normal rat embryo fibroblasts after the low UV dose (10 J/m^2) which again differs from the amount of induction we see in our mouse primary fibroblasts (a maximum of 3 to 4 fold induction over unirradiated p21 message levels). It is possible that the kinetics of the UV-induced activation of p21 transcription is significantly different in these two types of cells.

It seems that the p21 response after high UV doses that we see in the ERCC1-deficient mouse primary embryonic fibroblasts resembles that of normal cell lines from other species (human diploid fibroblasts, rat fibroblasts and rat embryonic fibroblasts) and differs from the normal p21 response seen in NER proficient mouse primary embryonic fibroblasts. It is possible that in mice, and rats and humans two pathways exist that deal with the response to high levels of UV damage. One of the pathways would involve the induction of p21 through an NER-related mechanism and it would be the main pathway in mice. The other pathway would play the most prominent role in humans and rats and would not depend on the induction of p21. It is possible that when ERCC1 is absent in mice their main response mechanism is impaired (thus the poor p21 response seen in ERCC1-deficient mouse primary embryonic fibroblasts) and mouse cells resort to the p21/NER-independent alternative pathway that is prominently active in rat and humans.

In summary, ERCC1-deficient mouse fibroblasts are more sensitive to UV in terms of their p53 response than their wild type counterparts, and appear to be normal in terms of the kinetics of its accumulation. On the other hand, p21, which has extensively been proposed to be transcriptionally activated in a p53 dependent manner under conditions of DNA damage, seems to be induced by UV via a p53-independent pathway in these fibroblasts. On the other hand, the basal levels of p21 are elevated in the ERCC1-deficient cells but they are drastically reduced (approximately a 10 fold reduction compared to wild-type levels) in those primary mouse fibroblasts deficient in p53 (both p53-/-ERCC1+/+ and double null cells) suggesting that under conditions of no DNA injury the amount of p21 message is likely to be under the control of p53.

Chapter 6

NER Studies on Hutchinson-Gilford Progeria Primary Fibroblasts

6.1 Introduction

No human disease has been associated with a defect in ERCC1. This could be because defects in ERCC1 are developmentally lethal or that an ERCC1 deficiency results in a pathology that differs totally from the expected defects in a NER-related syndrome and so has escaped identification up to now. On the other hand, some of the phenotypic characteristics of ERCC1-deficient mice are reminiscent of those of premature ageing, specially when the liver is considered.

Certain human syndromes such as Werner's syndrome (WS; adult progeria), Cockayne's syndrome (CS), Bloom's syndrome (BS) and ataxia telangiectasia (AT) have been studied as models for accelerated ageing¹. Studies carried out using cells derived from patients suffering from these premature ageing syndromes (WS, BS and AT) have found common patterns of chromosomal instability. The defect in WS has been found to lie in a DNA helicase (Yu et al., 1996). The BS gene has also been found to encode a helicase (Ellis et al., 1995) and AT cells, defective in the ATM gene function, have been proposed to fail to monitor DNA damage and activate DNA repair (Meyn, 1995; Jeggo et al., 1998). CS has also been associated with abnormalities in NER (Nance

¹ For a review see Dyer and Sinclair, 1998

and Berry, 1992; Venema et al., 1990). Thus, many of these syndromes support the idea that, at the DNA level, ageing results, in part, from an imbalance between DNA damage and repair.

Hutchinson–Gilford progeria is a very rare premature ageing human disease (1 affected individual born per 8 million people) of unknown aetiology (Dyer and Sinclair, 1998). Reports of patients suffering from this syndrome are scarce but the phenotypic description of some of the affected individuals and some studies performed on fibroblasts derived from them can be summarised as follows.

- Progeria patients present precocious senility, very early death from coronary artery disease (cardiac failure), growth retardation and failure to thrive (Abdenur et al., 1991), absence of subcutaneous fat, aged face and alopecia.
- At the molecular level, primary fibroblasts derived from some of these patients show increased elastin (Giro and Davidson, 1993; Colige et al., 1991b), fibronectin and collagen IV (Maquart et al., 1988; Colige et al., 1991b), altered response to EGF (Colige et al., 1991a), insulin resistance and hyperglycemia (Rosenbloom et al., 1983), high basal metabolic rates, low levels of insulin-like growth factor, high levels of hyaluronic acid (Zebrower et al., 1986; Brown, 1992), reduced insulin receptor protein levels, and most importantly in the context of the research carried out with ERCC1, decreased rate of DNA synthesis after UV irradiation (impaired capacity to repair DNA damage) (Sugita et al., 1995; Wang et al., 1990; Wang et al., 1991).

Some of the phenotypic aspects of the Hutchinson–Gilford patients are similar to those of the ERCC1–deficient mice (growth retardation, failure to thrive, premature death, absence of subcutaneous fat). Furthermore, at the cellular level the most interesting feature for us is the fact that these fibroblasts seem to have some impairment in their DNA repair capacity, which would be in accordance with a syndrome related to a DNA–repair deficiency.

In order to investigate if progeria could be due to a defect in ERCC1 studies on a primary fibroblast cell line derived from a Hutchinson–Gilford patient, AG08466, have

been carried out. Due to the lack of a suitable human primary fibroblast cell line, the primary control fibroblasts used in the experiments of p21 and p53 induction after UV damage were fetal mouse fibroblasts. For the rest of the experimental work a transformed wild type human cell line, MRC5, was used as a control.

6.2 Results

6.2.1 ERCC1 mRNA and protein levels are reduced in AG08466 cells.

To see if there were any major rearrangements in the genomic sequence of the ERCC1 gene, or if the transcript, or the ERCC1 proteins levels were affected in the progeria fibroblasts, Southern analysis, northern analysis and western analysis were carried out using DNA, RNA and protein extracts from the progeria fibroblasts (for more detail on the methodology refer to Materials and Methods). Protein concentrations were matched using the BCA protein concentration detection method, DNA loadings were tested by reprobing the Southern gels with a PrP probe (1.1 Kb SmaI/EcoRI PrP exon 3 probe (Moore et al., 1995)) and RNA loadings were also referred to a loading control, GAPDH.

Based on the genomic restriction sites described by van Duin *et al.*, a Southern blot was carried out using the following restriction enzymes: EcoRI, BamHI, BglII and SstI (van Duin et al., 1987) (see Fig. 6.1). Although it was clear from the first southern that there were no major rearrangements in the genomic sequence of ERCC1 in the progeria cells compared to the ERCC1 gene in the wild type MRC5 cells, there was an indication that the signal derived from the progeria DNA was weaker than that from the MRC5 DNA (see Fig. 6.1).

In order to assess whether this effect was due to a loading difference between the DNAs or to an actual copy loss of the ERCC1 gene in the progeria cells, a loading control using a PrP probe was performed on the southern blot. When the phosphorimage signals from the different bands corresponding to the progeria and the MRC5 fibroblasts were compared to their respective PrP signals, it was found out that the apparent difference in the intensity of the ERCC1 signal was in fact a loading artifact.

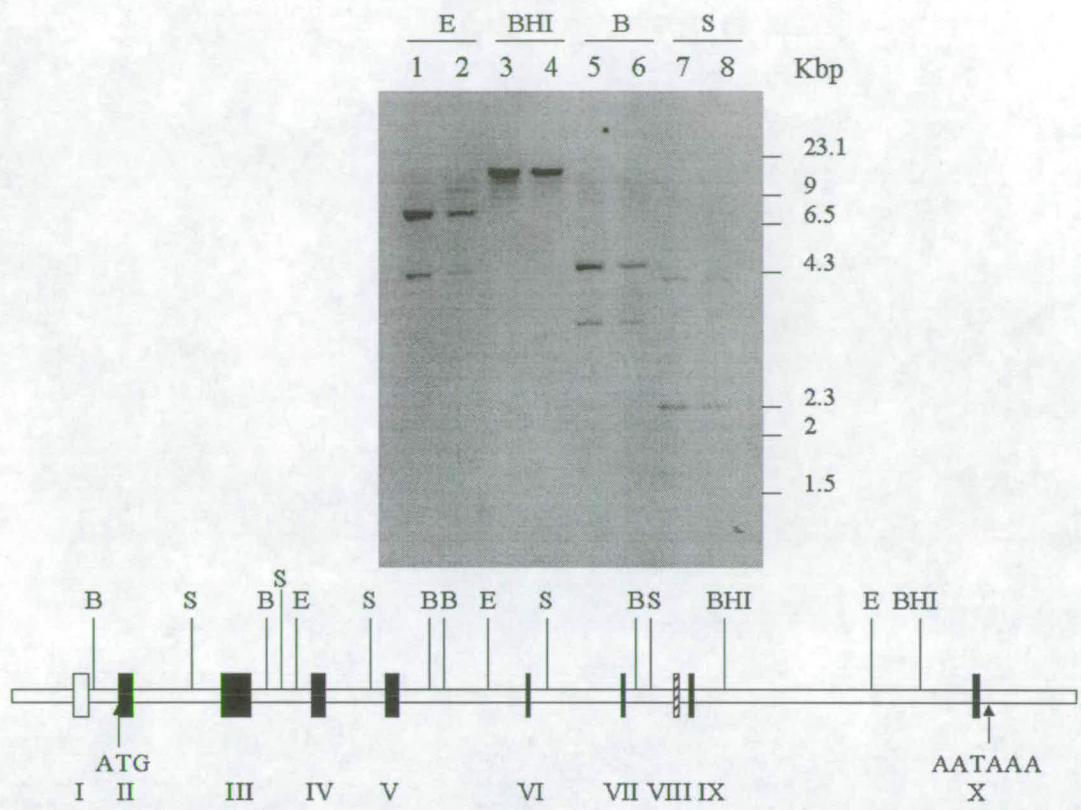


Figure 6.1: Southern blot of progeria (AG08466) and wild type (MRC5) genomic DNA probed with an ERCC1 probe (exon1–10). Lanes 1, 3, 5 and 7 correspond to MRC5 DNA. Lanes 2, 4, 6 and 8 correspond to progeria DNA. A schematic map corresponding to the exon–intron structure of the 15 Kb human ERCC1 gene with the approximate location of the cutting sites of the restriction enzymes used in this experiments is also shown. Exons are indicated by Roman numbers. Black boxes represent the coding exons and the shaded box is the differentially spliced exon VIII. This diagram has been adapted from van Duin *et al.*, 1987.

ERCC1 mRNA levels in the progeria fibroblasts were also determined by northern analysis. Again, MRC5 mRNA was used as a control, and a probe comprising ERCC1 exons 1–10 was used to probe the membranes. Fig. 6.2 A shows a typical progeria northern. Two samples belonging to the same RNA preparation were loaded per cell line. The GAPDH reprobe signals are shown in the same figure. The value of 1 for ERCC1/GAPDH signal is given to the average of the ratio of ERCC1/GAPDH signal for the two different loadings of MRC5 RNA. ERCC1/GAPDH ratios for the progeria samples is calculated relative to the ratio of the MRC5 control.

The results obtained using the northern analysis indicated that there were reduced levels of ERCC1 transcript in the progeria fibroblasts (approximately 1/2 of that in

wild type human fibroblasts). In order to confirm whether the levels of ERCC1 protein were reduced in the progeria cells, total protein was extracted from the AG08466 cells, as described in Materials and Methods, and 40 μ g of each lysate were loaded onto denaturing protein gels.

The western blots were probed with RW017, an antibody raised against human ERCC1, which also recognises hamster ERCC1 (see Materials and Methods, section 2.2.11, subsection 2.2.11 for details). Two CHO cell lines, one wild type (CHO9) and one containing mutant ERCC1 (CHO 43-3B), were included in the experiment in order to test the specificity of the RW017 antibody. It is known that due to the ERCC1 mutation in the CHO 43-3B cell line, the levels of ERCC1 are very low (barely detectable) in these cells (Hayashi et al., 1998).

Indeed, RW017 proved to be specific for ERCC1 in that it detected ERCC1 in hamster cells successfully (positive signal for CHO9 cells and non-detectable levels of ERCC1 protein in CHO 43-3B cells). When the human cell lines were used, a reduction in the ERCC1 protein levels was detected in the progeria cell line, which supported the result obtained with the northern analysis.

Since both the transcript levels and the protein levels of ERCC1 seemed to be at best only reduced in the progeria fibroblasts, and there was no sign of a major rearrangement in the genomic sequence of the gene, it was decided to look for possible mutations in the coding sequence of ERCC1. This was done by sequencing overlapping ERCC1 PCR fragments obtained from total progeria cDNA. As a control, the same procedure was carried out using total MRC5 cDNA (see Material and Methods for more details). Table 6.1 shows the different primers used and the combinations they were used in to obtain the complete ERCC1 cDNA sequence.

Once the different DNA sequences from each PCR reaction were obtained, they were manipulated using the GCG package. Overlapping stretches of DNA were identified and the sequences were edited. Once the whole cDNA sequence was obtained and edited for both MRC5 and progeria cells, they were compared to each other and to the existing GenBank human ERCC1 cDNA sequence, using the 'LineUp' program. The result obtained is presented in Table 6.2. When the ERCC1 coding sequence from

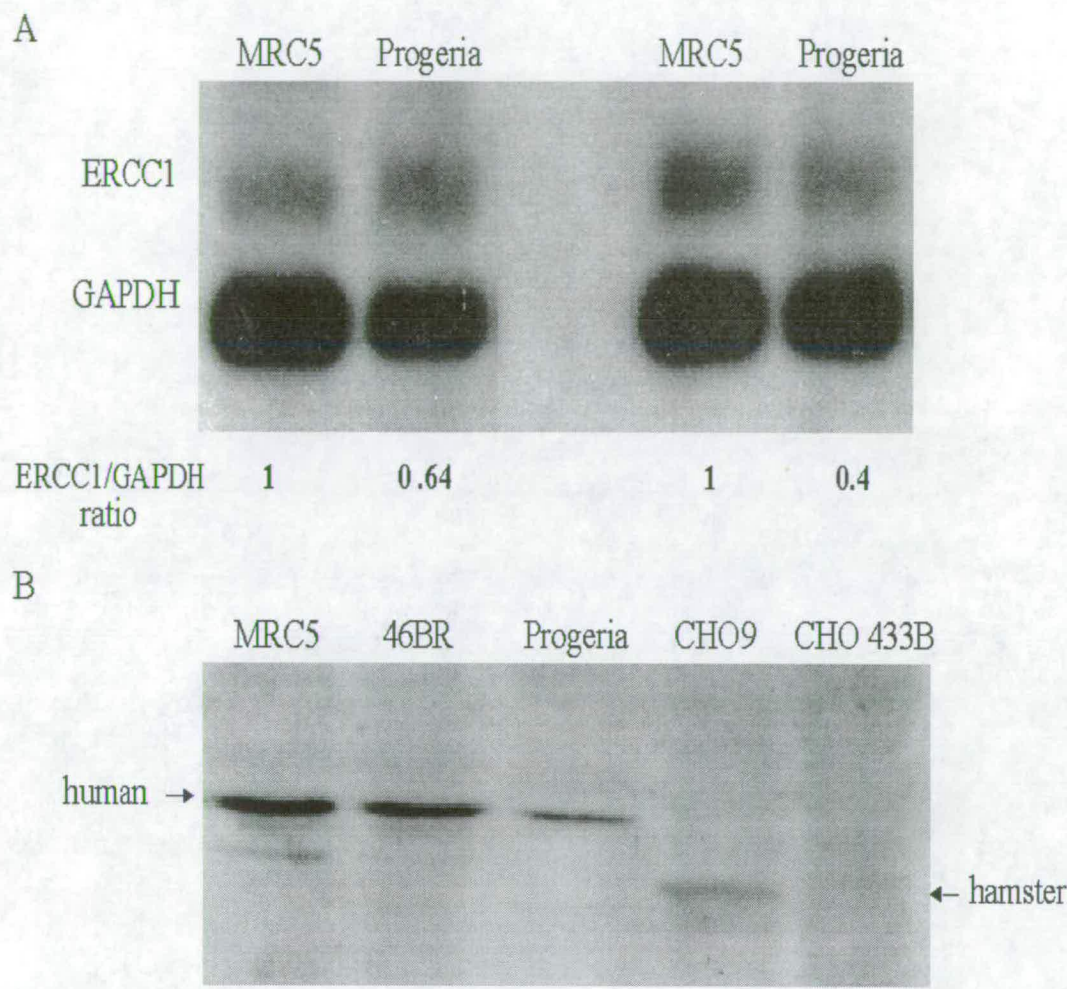


Figure 6.2: (A) ERCC1 mRNA levels in progeria and wild type transformed human fibroblasts. Two separate experiments are shown. Levels of ERCC1 message are expressed as ratios of ERCC1/GAPDH signal. (B) ERCC1 protein levels in progeria and human fibroblasts. As controls, to test the antibody (RW017, see Materials and Methods) specificity, 2 CHO lines are shown. CHO 43-3B (ERCC1-mutant), levels of ERCC1 in this line range from very low to undetectable; wild type CHO9, normal levels of ERCC1. As controls for the ERCC1 protein levels in human fibroblasts, MRC5 (wild type) and 46BR (Ligase I-mutant). Human protein concentrations were matched to 40 μ g protein/lane (see Materials and Methods, section 2.2.11, subsection 2.2.11 for more detail).

the progeria cells was compared to both the MRC5 ERCC1 coding sequence and the GenBank ERCC1 cDNA sequence, no mutations were found (see Table 6.2).

In summary, there are no mutations in the coding sequence of ERCC1 in progeria cells

and no major rearrangements in its genomic sequence. However, there is a suggestion that ERCC1 transcript and protein levels are reduced in these cells compared to wild type human fibroblasts.

Is this reduction in the ERCC1 protein levels something that is particular to the ERCC1 protein in these cells or is it the case that these cells show reduced levels of other DNA repair-related proteins too? In order to answer this question and to see if progeria cells showed an ERCC1 protein-specific defect we devised the experiments detailed in the next section.

6.2.2 Ligase I, III and IV protein levels are reduced in AG08466 cells but XPF protein levels are normal.

Protein levels corresponding to a number of other proteins were investigated following the same procedure as the one detailed for the ERCC1 protein. The antibodies used were chosen firstly, because of their availability in the laboratory and secondly, they were chosen so that an array of repair-related proteins could be assayed in relation to a proper control protein whose expression should be normal in progeria fibroblasts.

The chosen ‘control’ protein was Vimentin, an intermediate filament protein which is normally used as a marker for fibroblasts. The rest of the proteins studied were Ligases I, III and IV and XPF.

We were interested in the expression of XPF because this protein forms a complex with ERCC1 to create a sequence-specific endonuclease and the levels of ERCC1 and

<i>Name</i>	<i>Sequence (5'→3')(Exon)</i>	<i>F/R</i>	<i>Primer combination</i>
C8438	CCCTTGAGGCTCCAAGACCAGCAGG (E1)	F	C8438-C8439
A9568	GATGGACCCTGGGAAGGACAAAGAGG (E2)	F	A9568-A9570
C8439	CCCTTGAGGCTCCAAGACCAGCAGG (E2)	R	C8439-C8438
A9569	CCGTACTGAAGTTCGTGCGCAACG (E4)	F	A9569-A9570&A9571
A9570	GCAAGGCGAAGTTCTTCCCCAGG (E5)	R	A9570-A9568&A9569
A9571	TCATCAGGGTACTTTCAAGAAGGG (E10)	R	A9571-A9569

Table 6.1: The primers in the table are listed in the same order as they are present on the cDNA sequence (Table 6.2). The polarity of the primer is represented by the letters F (forward) and R (reverse). The primer combination(s) that each primer has been used in is also shown.

	1				50
hsercc1.frg	AAGTGCTGCG	AGCCCTGGGC	CACGCTGGCC	GTGCTGGCAG	TGGGCCGCCT
mrc5ercc1.frg
progercc1.frg
consensus
	51				100
hsercc1.frg	CGATCCCTCT	GCAGTCTTTC	CCTTGAGGCT	CCAAGACCAG	CAGGTGAGGC
mrc5ercc1.frg	CAGGTGAGGC
progercc1.frg	CAGGTGAGGC
consensus	CAGGTGAGGC
	101				150
hsercc1.frg	CTCGCGGCGC	TGAAACCGTG	AGGCCCGGAC	CACAGGCTCC	AGATGGACCC
mrc5ercc1.frg	CTCGCGGCGC	TGAAACCGTG	AGGCCCGGAC	CACAGGCTCC	AGATGGACCC
progercc1.frg	CTCGCGGCGC	TGAAACCGTG	AGGCCCGGAC	CACAGGCTCC	AGATGGACCC
consensus	CTCGCGGCGC	TGAAACCGTG	AGGCCCGGAC	CACAGGCTCC	AGATGGACCC
	151				200
hsercc1.frg	TGGGAAGGAC	AAAGAGGGGG	TGCCCCAGCC	CTCAGGGCCG	CCAGCAAGGA
mrc5ercc1.frg	TGGGAAGGAC	AAAGAGGGGG	TGCCCCAGCC	CTCAGGGCCG	CCAGCAAGGA
progercc1.frg	TGGGAAGGAC	AAAGAGGGGG	TGCCCCAGCC	CTCAGGGCCG	CCAGCAAGGA
consensus	TGGGAAGGAC	AAAGAGGGGG	TGCCCCAGCC	CTCAGGGCCG	CCAGCAAGGA
	201				250
hsercc1.frg	AGAAATTTGT	GATACCCCTC	GACGAGGATG	AGGTCCCTCC	TGGAGTGGCC
mrc5ercc1.frg	AGAAATTTGT	GATACCCCTC	GACGAGGATG	AGGTCCCTCC	TGGAGTGGCC
progercc1.frg	AGAAATTTGT	GATACCCCTC	GACGAGGATG	AGGTCCCTCC	TGGAGTGGCC
consensus	AGAAATTTGT	GATACCCCTC	GACGAGGATG	AGGTCCCTCC	TGGAGTGGCC
	251				300
hsercc1.frg	AAGCCCTTAT	TCCGATCTAC	ACAGAGCCTT	CCCAGTGTGG	ACACCTCGGC
mrc5ercc1.frg	AAGCCCTTAT	TCCGATCTAC	ACAGAGCCTT	CCCAGTGTGG	ACACCTCGGC
progercc1.frg	AAGCCCTTAT	TCCGATCTAC	ACAGAGCCTT	CCCAGTGTGG	ACACCTCGGC
consensus	AAGCCCTTAT	TCCGATCTAC	ACAGAGCCTT	CCCAGTGTGG	ACACCTCGGC

	301				350
hserccl.frg	CCAGGCGGCC	CCTCAGACCT	ACGCCGAATA	TGCCATCTCA	CAGCCTCTGG
mrc5erccl.frg	CCAGGCGGCC	CCTCAGACCT	ACGCCGAATA	TGCCATCTCA	CAGCCTCTGG
progerccl.frg	CCAGGCGGCC	CCTCAGACCT	ACGCCGAATA	TGCCATCTCA	CAGCCTCTGG
consensus	<i>CCAGGCGGCC</i>	<i>CCTCAGACCT</i>	<i>ACGCCGAATA</i>	<i>TGCCATCTCA</i>	<i>CAGCCTCTGG</i>
	351				400
hserccl.frg	AAGGGGCTGG	GGCCACGTGC	CCCACAGGGT	CAGAGCCCCT	GGCAGGAGAG
mrc5erccl.frg	AAGGGGCTGG	GGCCACGTGC	CCCACAGGGT	CAGAGCCCCT	GGCAGGAGAG
progerccl.frg	AAGGGGCTGG	GGCCACGTGC	CCCACAGGGT	CAGAGCCCCT	GGCAGGAGAG
consensus	<i>AAGGGGCTGG</i>	<i>GGCCACGTGC</i>	<i>CCCACAGGGT</i>	<i>CAGAGCCCCT</i>	<i>GGCAGGAGAG</i>
	401				450
hserccl.frg	ACGCCCAACC	AGGCCCTGAA	ACCCGGGGCA	AAATCCAACA	GCATCATTGT
mrc5erccl.frg	ACGCCCAACC	AGGCCCTGAA	ACCCGGGGCA	AAATCCAACA	GCATCATTGT
progerccl.frg	ACGCCCAACC	AGGCCCTGAA	ACCCGGGGCA	AAATCCAACA	GCATCATTGT
consensus	<i>ACGCCCAACC</i>	<i>AGGCCCTGAA</i>	<i>ACCCGGGGCA</i>	<i>AAATCCAACA</i>	<i>GCATCATTGT</i>
	451				500
hserccl.frg	GAGCCCTCGG	CAGAGGGGCA	ATCCCGTACT	GAAGTTCGTG	CGCAACGTGC
mrc5erccl.frg	GAGCCCTCGG	CAGAGGGGCA	ATCCCGTACT	GAAGTTCGTG	CGCAACGTGC
progerccl.frg	GAGCCCTCGG	CAGAGGGGCA	ATCCCGTACT	GAAGTTCGTG	CGCAACGTGC
consensus	<i>GAGCCCTCGG</i>	<i>CAGAGGGGCA</i>	<i>ATCCCGTACT</i>	<i>GAAGTTCGTG</i>	<i>CGCAACGTGC</i>
	501				550
hserccl.frg	CCTGGGAATT	TGGCGACGTA	ATTCCCGACT	ATGTGCTGGG	CCAGAGCACC
mrc5erccl.frg	CCTGGGAATT	TGGCGACGTA	ATTCCCGACT	ATGTGCTGGG	CCAGAGCACC
progerccl.frg	CCTGGGAATT	TGGCGACGTA	ATTCCCGACT	ATGTGCTGGG	CCAGAGCACC
consensus	<i>CCTGGGAATT</i>	<i>TGGCGACGTA</i>	<i>ATTCCCGACT</i>	<i>ATGTGCTGGG</i>	<i>CCAGAGCACC</i>
	551				600
hserccl.frg	TGTGCCCTGT	TCCTCAGCCT	CCGCTACCAC	AACCTGCACC	CAGACTACAT
mrc5erccl.frg	TGTGCCCTGT	TCCTCAGCCT	CCGCTACCAC	AACCTGCACC	CAGACTACAT
progerccl.frg	TGTGCCCTGT	TCCTCAGCCT	CCGCTACCAC	AACCTGCACC	CAGACTACAT
consensus	<i>TGTGCCCTGT</i>	<i>TCCTCAGCCT</i>	<i>CCGCTACCAC</i>	<i>AACCTGCACC</i>	<i>CAGACTACAT</i>

	601				650
hserccl.frg	CCATGGGCGG	CTGCAGAGCC	TGGGGAAGAA	CTTCGCCTTG	CGGGTCCTGC
mrc5erccl.frg	CCATGGGCGG	CTGCAGAGCC	TGGGGAAGAA	CTTCGCCTTG	CGGGTCCTGC
progerccl.frg	CCATGGGCGG	CTGCAGAGCC	TGGGGAAGAA	CTTCGCCTTG	CGGGTCCTGC
consensus	<i>CCATGGGCGG</i>	<i>CTGCAGAGCC</i>	<i>TGGGGAAGAA</i>	<i>CTTCGCCTTG</i>	<i>CGGGTCCTGC</i>
	651				700
hserccl.frg	TTGTCCAGGT	GGATGTGAAA	GATCCCCAGC	AGGCCCTCAA	GGAGCTGGCT
mrc5erccl.frg	TTGTCCAGGT	GGATGTGAAA	GATCCCCAGC	AGGCCCTCAA	GGAGCTGGCT
progerccl.frg	TTGTCCAGGT	GGATGTGAAA	GATCCCCAGC	AGGCCCTCAA	GGAGCTGGCT
consensus	<i>TTGTCCAGGT</i>	<i>GGATGTGAAA</i>	<i>GATCCCCAGC</i>	<i>AGGCCCTCAA</i>	<i>GGAGCTGGCT</i>
	701				750
hserccl.frg	AAGATGTGTA	TCCTGGCCGA	CTGCACATTG	ATCCTCGCCT	GGAGCCCCGA
mrc5erccl.frg	AAGATGTGTA	TCCTGGCCGA	CTGCACATTG	ATCCTCGCCT	GGAGCCCCGA
progerccl.frg	AAGATGTGTA	TCCTGGCCGA	CTGCACATTG	ATCCTCGCCT	GGAGCCCCGA
consensus	<i>AAGATGTGTA</i>	<i>TCCTGGCCGA</i>	<i>CTGCACATTG</i>	<i>ATCCTCGCCT</i>	<i>GGAGCCCCGA</i>
	751				800
hserccl.frg	GGAAGCTGGG	CGGTACCTGG	AGACCTACAA	GGCCTATGAG	CAGAAACCAG
mrc5erccl.frg	GGAAGCTGGG	CGGTACCTGG	AGACCTACAA	GGCCTATGAG	CAGAAACCAG
progerccl.frg	GGAAGCTGGG	CGGTACCTGG	AGACCTACAA	GGCCTATGAG	CAGAAACCAG
consensus	<i>GGAAGCTGGG</i>	<i>CGGTACCTGG</i>	<i>AGACCTACAA</i>	<i>GGCCTATGAG</i>	<i>CAGAAACCAG</i>
	801				850
hserccl.frg	CGGACCTCCT	GATGGAGAAG	CTAGAGCAGG	ACTTCGTCTC	CCGGGTGACT
mrc5erccl.frg	CGGACCTCCT	GATGGAGAAG	CTAGAGCAGG	ACTTCGTCTC	CCGGGTGACT
progerccl.frg	CGGACCTCCT	GATGGAGAAG	CTAGAGCAGG	ACTTCGTCTC	CCGGGTGACT
consensus	<i>CGGACCTCCT</i>	<i>GATGGAGAAG</i>	<i>CTAGAGCAGG</i>	<i>ACTTCGTCTC</i>	<i>CCGGGTGACT</i>
	851				900
hserccl.frg	GAATGTCTGA	CCACCGTGAA	GTCAGTCAAC	AAAACGGACA	GTCAGACCCT
mrc5erccl.frg	GAATGTCTGA	CCACCGTGAA	GTCAGTCAAC	AAAACGGACA	GTCAGACCCT
progerccl.frg	GAATGTCTGA	CCACCGTGAA	GTCAGTCAAC	AAAACGGACA	GTCAGACCCT
consensus	<i>GAATGTCTGA</i>	<i>CCACCGTGAA</i>	<i>GTCAGTCAAC</i>	<i>AAAACGGACA</i>	<i>GTCAGACCCT</i>

	901				950
hsercc1.frg	CCTGACCACA	TTTGGATCTC	TGGAACAGCT	CATCGCCGCA	TCAAGAGAAG
mrc5ercc1.frg	CCTGACCACA	TTTGGATCTC	TGGAACAGCT	CATCGCCGCA	TCAAGAGAAG
progercc1.frg	CCTGACCACA	TTTGGATCTC	TGGAACAGCT	CATCGCCGCA	TCAAGAGAAG
consensus	<i>CCTGACCACA</i>	<i>TTTGGATCTC</i>	<i>TGGAACAGCT</i>	<i>CATCGCCGCA</i>	<i>TCAAGAGAAG</i>
	951				1000
hsercc1.frg	ATCTGGCCTT	ATGCCCAGGC	CTGGGCCCTC	AGAAAGCCCG	GAGGCTGTTT
mrc5ercc1.frg	ATCTGGCCTT	ATGCCCAGGC	CTGGGCCCTC	AGAAAGCCCG	GAGGCTGTTT
progercc1.frg	ATCTGGCCTT	ATGCCCAGGC	CTGGGCCCTC	AGAAAGCCCG	GAGGCTGTTT
consensus	<i>ATCTGGCCTT</i>	<i>ATGCCCAGGC</i>	<i>CTGGGCCCTC</i>	<i>AGAAAGCCCG</i>	<i>GAGGCTGTTT</i>
	1001				1050
hsercc1.frg	GATGTCCTGC	ACGAGCCCTT	CTTGAAAGTA	CCCTGATGAC	CCCAGCTGCC
mrc5ercc1.frg	GATGTCCTGC	ACGAGCCCTT	CTTGAAAGTA	CCCTGATGAC	CCCAGCTGCC
progercc1.frg	GATGTCCTGC	ACGAGCCCTT	CTTGAAAGTA	CCCTGATGAC	C.....
consensus	<i>GATGTCCTGC</i>	<i>ACGAGCCCTT</i>	<i>CTTGAAAGTA</i>	<i>CCCTGATGAC</i>	<i>CCCAGCTGCC</i>
	1051				1097
hsercc1.frg	AAGGAAACCC	CCAGTGTAAT	AATAAATCGT	CCTCCCAGGC	CAGGCTC.....
mrc5ercc1.frg	A.....
progercc1.frg
consensus	<i>AAGGAAACCC</i>	<i>CCAGTGTAAT</i>	<i>AATAAATCGT</i>	<i>CCTCCCAGGC</i>	<i>CAGGCTC.....</i>

Table 6.2: ERCC1 cDNA sequence in progeria primary fibroblasts and transformed wild type MRC5 fibroblasts. The human ERCC1 cDNA sequence hsercc1 was extracted from GenBank and used as a reference to check the obtained sequences. The total ERCC1 cDNA sequence from both fibroblast cell lines was obtained as stated in Materials and Methods, section 2.2.9. The position of PCR primers is shown on the sequence extracted from GenBank. Forward PCR primers are shown in blue; reverse primers are shown in green; START and STOP codons are shown in red. The consensus sequence obtained with the program LineUp (GCG package) is shown in slanted font. For a detailed account of the combinations of primers used in order to obtain the various PCR fragments necessary to cover the entire cDNA sequence see Table 6.1.

XPF have been shown to be related to one another, i.e., ERCC1 levels are reduced in XPF-deficient cells (Sijbers et al., 1996a).

The Ligases offered an external control group in that they are involved in repair processes, but also in replication and other cellular mechanisms, which allowed us to investigate the functional status of progeria cells in a number of cellular activities. DNA Ligase I is specifically involved in replication and it also participates in base excision repair. The DNA Ligase III- α isoform, which is ubiquitously expressed, forms a complex with the DNA single-strand break repair protein XRCC1 and the DNA ligase III- β isoform has been proposed to play a role in meiotic recombination (Tomkinson and Levin, 1997). Ligase IV has been shown to form a complex with XRCC4 and its role may be to carry out the final steps of V(D)J recombination and joining of DNA ends (Grawunder et al., 1997). Yeast DNA ligase IV has been shown to mediate non-homologous DNA end joining (Wilson et al., 1997).

The results obtained are summarised in Figure 6.3. It was found that levels of Ligase I, Ligase III and Ligase IV proteins were reduced, although normal levels of XPF and Vimentin were present in these fibroblasts (see Fig. 6.3). This points towards a very complicated aetiology of the progeroid syndrome. It is possible that the levels of Ligase I are found to be reduced in these cells because their replicative capacity is extremely reduced, but no easy explanation is found for the fact that also Ligase III and Ligase IV levels are barely detectable using this assay. Ligase IV signal was also very weak in the control fibroblasts so it cannot be ruled out that the low levels of Ligase IV in progeria cells may be due to technical limitation of the immunoblotting procedure using the anti-Ligase IV antibody.

It is interesting that Vimentin levels in progeria cells are equivalent to the levels detected in wild type transformed MRC5 fibroblasts. This rules out the possibility that progeria cells, due to their general growth and reproductive impairment, present low levels of a wide range of molecules.

The other protein that seems to maintain wild type-like levels in the progeria fibroblasts is XPF. This result is somehow unexpected since, as has been mentioned before, ERCC1 and XPF form a complex and levels of ERCC1 and XPF have been shown to

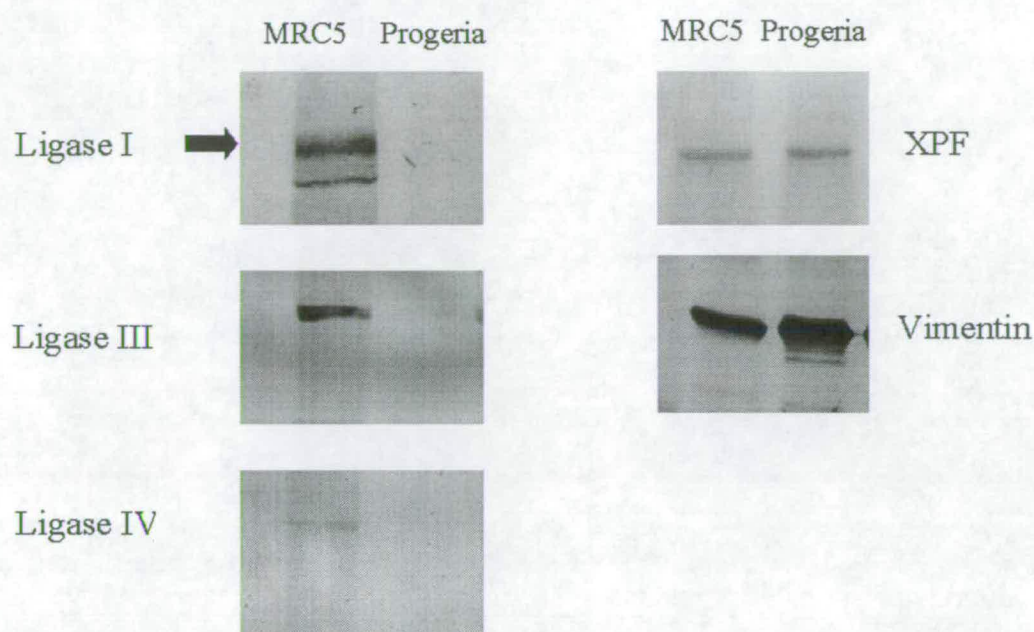


Figure 6.3: Different proteins levels in progeria and wild type transformed human fibroblasts (MRC5). Protein concentrations were matched to 40 μg protein/lane (see Materials and Methods, section 2.2.11 for more detail). For more information and the details concerning the origin and specificity of the antibodies refer to Materials and Methods, section 2.1.1.

be correlated in XPF-deficient cells (van Vuuren et al., 1995; Yagi et al., 1997). It would be interesting to test the possibility that these cells are defective in some recombinational processes. However, the limitations imposed by the poor growth presented by these cells and their extremely long population doubling time, make it difficult to carry out demanding experimental procedures on these cells.

6.2.3 AG08466 cells fail to induce p53 and p21 in response to UV irradiation.

There are some reports in the literature that study the response of progeria fibroblasts to UV. These reports show that some progeria cells have a reduced amount of unscheduled DNA synthesis after UV irradiation, which has led to the hypothesis that these cells may have a defect in the DNA damage-response mechanisms, and more specifically in the repair of UV excision damage (Sugita et al., 1995; Wang et al., 1990;

Wang et al., 1991). In addition to this, the experiments detailed above suggest that a wide range of metabolic functions may be compromised in these cells, from replication to DNA single-strand break repair.

Another aspect of the cellular metabolism that could give an indication of the status of these cells in relation to the DNA damage repair mechanisms is the capacity of progeria fibroblasts to detect the damage inflicted to their DNA. p53 and p21 have been shown to be part of the DNA damage response machinery, and, in particular, a great deal of studies have shown that p53 protein is readily stabilised in response to UV (see Chapter 5), specially in cell lines with defects in their DNA repair machinery.

In order to investigate the p53 and the p21 response of the progeria fibroblasts to UV damage, AG08466 cells were UV-irradiated with a dose of 20J/m² and/or 50J/m². The p53 and p21 response to UV was monitored using either immunoblotting to monitor p53 accumulation, or northern analysis, using a p21 PCR probe to detect transcriptional induction of p21 message. Since no human wild type primary fibroblasts were available in the laboratory at the time the experiments were carried out, mouse wild type primary fibroblasts were used as controls for the induction of p53 stabilisation and p21 transcription after UV.

A summary of the results obtained is shown in Figs. 6.4 A and B. In Fig. 6.4 A, three different transformed human cell lines were used as controls for the migration pattern of human p53 protein since there is a slight difference in the migration patterns of human and mouse p53. All the protein concentrations used for this experiment were matched to 40 μ g per lane and the samples were taken 24-30 hours after UV irradiation, to allow for high p53 levels to be present in the cells and also to allow for a possible delay in the kinetics of p53 stabilisation in the progeria cells (the p53 response in human primary fibroblasts after high UV doses is known to remain elevated 24 hours after UV irradiation (Dumaz et al., 1998)).

Overall, p53 levels in the unirradiated progeria and mouse primary fibroblasts were very low and barely detectable by this assay, which is to be expected in normal primary fibroblasts. However, after both doses of UV irradiation (20 and 50 J/m²), we failed to see any sign of p53 stabilisation in the progeria fibroblasts. This is contrast with the

expected response in cells that seem to have a defect in the DNA-repair mechanism (Lu and Lane, 1993; Sanchez and Elledge, 1995). On the other hand, and as expected, wild type mouse primary fibroblasts show a prolonged p53 stabilisation (samples were taken 30 hours after UV irradiation with a dose of 20 J/m²).

To see if p21 transcriptional induction was also compromised, we performed northern analysis of the levels of p21 transcript after a UV dose of 50 J/m² in the progeria fibroblasts and compared this response to the response of mouse primary fibroblasts after a UV dose of 20 J/m². Both human and mouse RNA samples were collected 24-30 hours after UV-irradiation. The results from this experiment are shown in Fig. 6.4 B.

It is apparent from this experiment that mouse primary fibroblasts show the predicted response to UV damage, i.e., they have a 3-fold induction of p21 transcript 30 hours after UV-irradiation compared to the unirradiated mouse controls. Contrary to this, progeria primary fibroblasts do not show a significant induction of p21 message levels even after a more dramatic UV challenge (50 J/m² compared to 20 J/m² in the case of the mouse cells). It is also noticeable that the levels of p21 transcript detected in unirradiated mouse primary fibroblasts are twice those seen in the unirradiated progeria fibroblasts (see Discussion).

Despite this apparent difference in the p53 and p21 responses of these two cell lines, it has to be taken into account that mouse and human cells respond differently to UV damage, both in terms of the mechanisms that deal with the overcoming of the DNA damage-induced cell cycle arrest and in the kinetics of DNA damage removal (see Chapter 4). Mice are less efficient in the removal of UV-induced DNA damage, which makes them more tolerant to the presence of unrepaired DNA, and in turn makes the DNA damage recognition mechanisms more prone to suffer long term induction. This would explain the prolonged p53 stabilisation and long term induction of p21 mRNA transcription.

However, these differences between mouse and human cells do not account for the lack of any apparent UV-induced response in the progeria cells in terms of p53 accumulation or p21 transcriptional activation. Human cells are known to arrest cell cycle progression

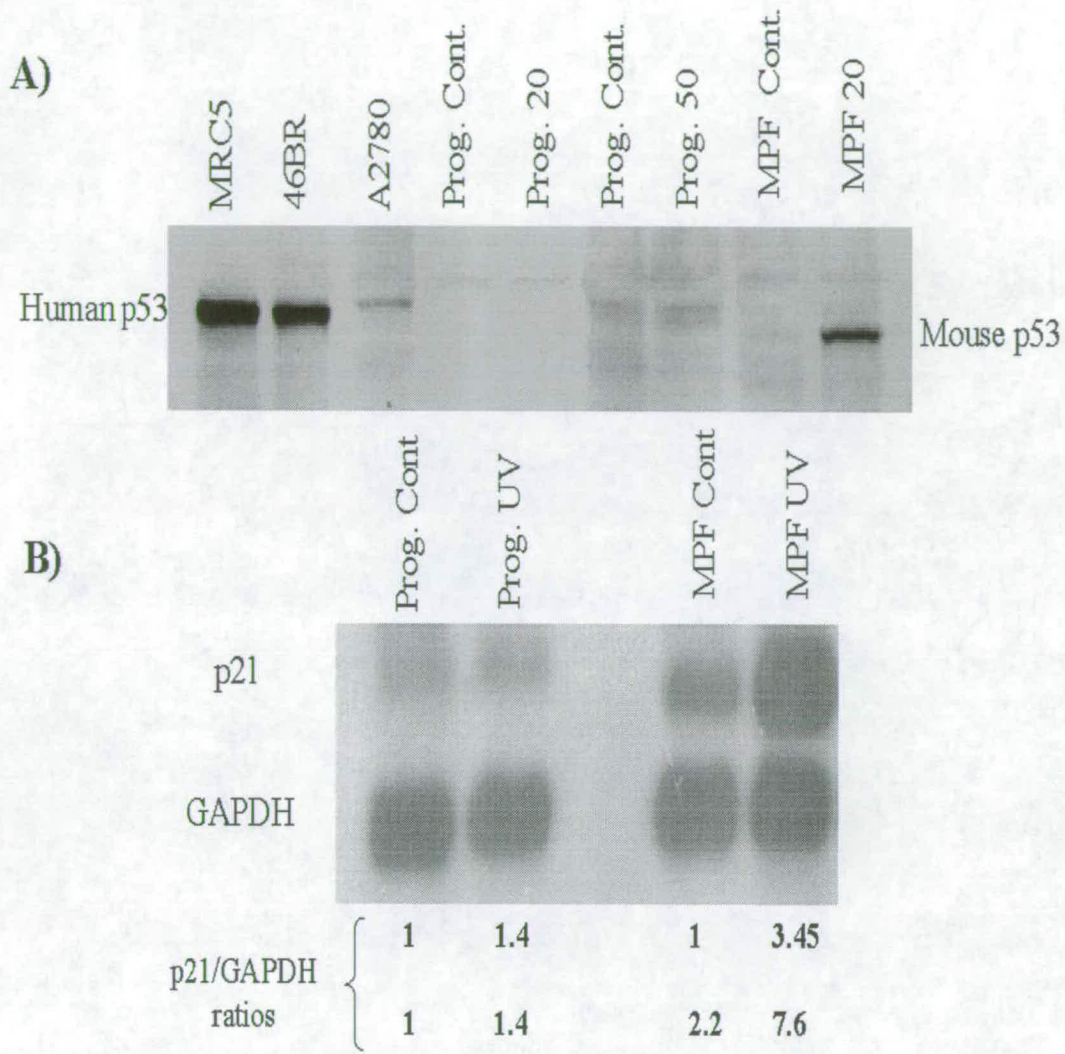


Figure 6.4: A) p53 stabilisation in progeria fibroblasts after UV (20 and 50 J/m²). 1) MRC5, transformed human fibroblasts; 2)46BR, transformed human fibroblasts, Ligase I-deficient; 3) A2780, human ovarian carcinoma cell line; 4) progeria unirradiated; 5) progeria irradiated (20 J/m²); 6) progeria unirradiated; 7) progeria irradiated (50 J/m²); 8) MPF (mouse primary fibroblasts) unirradiated; 9) MPF (mouse primary fibroblasts) irradiated (20 J/m²). B) p21 induction after UV irradiation in progeria and wild type primary mouse fibroblasts. p21 levels are expressed as p21/GAPDH ratios. 1) progeria unirradiated; 2) progeria irradiated (50 J/m²); 3) MPF (mouse primary fibroblasts) wild type unirradiated; 4) MPF (mouse primary fibroblasts) wild type irradiated (20 J/m²). The top p21/GAPDH ratio has been calculated taking each unirradiated control as a separate value of 1, to indicate induction of p21 in each of the primary fibroblast cell lines. The bottom p21/GAPDH ratio has been calculated taking the p21 mRNA levels in unirradiated progeria fibroblasts alone as 1, to indicate differences in the levels of basal p21 message between unirradiated mouse and progeria primary fibroblasts.

after UV irradiation in order to allow DNA repair to take place (Sanchez and Elledge, 1995). This arrest is known to be prolonged and p53 has been implicated in this mechanism.

p21 is an effector of p53 and accordingly, its mRNA levels are elevated through its transcriptional activation by p53. p21 is known to be involved in the arrest of the cell cycle that is seen in human cells after UV. It would be expected that after UV damage has occurred, both p53 and p21 responses would be activated in the irradiated cells. Reports exist, however, that p21 protein induction is inhibited in normal human diploid fibroblasts after high UV doses (60 J/m^2) despite accumulation of p53 protein levels (Reinke and Lozano, 1997). Reinke and Lozano suggest that after such high UV doses the levels of DNA damage are so high that even transcription processes are arrested. This could also be the case for the progeria fibroblasts but, as discussed in the previous chapter, these authors looked at the p21 response up to 12 hours after UV irradiation and the extent of the inhibition of the p21 response cannot be determined for longer periods of time post-irradiation.

Overall, it seems that AG08466 progeria cells fail to detect DNA damage and thus they fail to respond to it, at least in a way that involves a p53 and, possibly, a subsequent p21 response.

6.3 Discussion

DNA repair defects have been proposed to have an effect on the ageing process of organisms through their involvement in the processing of DNA damage, and their role in preventing genetic instability (Dyer and Sinclair, 1998; Kirkwood, 1989). Conversely, ageing in humans is associated with decreasing ability of cultured skin and blood cells to process new UV-induced DNA damage (Moriwaki et al., 1996).

The ERCC1 knock-out model presents some characteristics reminiscent of premature ageing, and since there has not been a human disease associated with a defect in this gene, it seemed logical to investigate the possibility of it being involved in the aetiology of a premature ageing syndrome. Defects in two different helicases and different genes involved in DNA repair have been shown to be responsible for some premature ageing

diseases such as Werner's syndrome, Bloom's syndrome, Cockayne's syndrome and ataxia telangiectasia. There is however, another extremely rare premature ageing disease whose aetiology has not been identified, Hutchinson–Gilford neonatal progeria.

Cells derived from patients suffering from this disease have already been shown to have impaired DNA repair capacity (Sugita et al., 1995; Wang et al., 1990; Wang et al., 1991) and defects in the timing of the DNA repair process relative to replication (Lipman et al., 1989).

The progeria cell line AG08466 has been used in this work in order to assess the status of the ERCC1 gene and some other DNA repair-related proteins. ERCC1 protein and mRNA levels are reduced in these cells, although no major gene rearrangements as shown by Southern blot have been found. Also, no mutations in the ERCC1 cDNA of these cells have been detected.

XPF and Vimentin proteins are found in levels equivalent to wild type. Since XPF and ERCC1 form a complex that functions as a structure-specific endonuclease it seems of interest that the levels of ERCC1 are reduced in progeria cells while XPF levels are apparently normal. Indeed, XPF has a prominent role in the stabilisation of ERCC1 protein as found by mutation studies of ERCC1 that affect the domain of XPF–ERCC1 interaction. Any mutation, either in XPF or ERCC1, that impedes the interaction between these two proteins decreases the half-life of ERCC1 protein in the cell, resulting in reduced ERCC1 protein amounts in the affected cells (Sijbers et al., 1996a). No mutations have been found in the ERCC1 cDNA in progeria cells but the possibility remains that XPF may be mutated in these cells. However, it has been reported that XPF protein levels are reduced to 5% of those in normal cells in XPF-deficient fibroblasts (Brookman et al., 1996; Yagi et al., 1997). Therefore, the fact that XPF levels appear to be normal in progeria cells argues against a mutation in this gene.

The levels of other proteins such as Ligases I, III and IV are also reduced. Reduced levels of Ligase I can be explained by the fact that this protein is present in lower quantities in slow growing cells (Lindahl and Barnes, 1992). The absence of detectable levels of the other ligases may have some implications for the aetiology of progeroid

cells. As mentioned in the previous section, Ligase IV seems to be involved in non-homologous DNA end joining (Wilson et al., 1997) and the Ligase III- α isoform seems to play an important role in DNA single-strand break repair, along with XRCC1, with which it forms a complex. The possible defects that would be derived from an impaired expression of these proteins, would have serious repercussions in the DNA metabolism of the cell. However, these reduced levels of ligases are more likely to be the effect of the progeroid syndrome rather than its cause.

The response of these progeria cells to UV-induced damage was also studied by measuring the induction of both p53 protein stabilisation (after 20 and 50 J/m²) and p21 mRNA transcription (after 50J/m²). No obvious induction of either was found to occur in the progeroid cells, which points to a defect in this cell line in the recognition of DNA damage. It has been observed previously that human cells are particularly proficient at inducing p53 and p21 in response to DNA damage. This induction is both fast and prolonged in order to allow the DNA to be repaired before replication takes place (Sanchez and Elledge, 1995; Lu and Lane, 1993). Lipman *et al.*, 1989, suggest that temporal control of DNA repair (which in part is regulated by p53 and p21) is impaired in progeria cells, which supports the findings presented here. If this regulatory mechanism is missing in these cells it is reasonable to speculate that the acceleration of age-associated changes observed in progeria may relate to an inability of the cells to optimally repair DNA damage before the onset of DNA synthesis. It is also of interest to note that 2 out of 11 cell lines derived from Bloom's syndrome patients have been reported to show no p53 accumulation after UV irradiation (Lu and Lane, 1993). These cells also show an aberrant response following X-ray. We have not performed any X-ray irradiation experiments with these cells but the experiment should prove of interest.

The basal levels of p21 mRNA were also found to be reduced when compared to mouse p21 transcript basal levels. One possible explanation for this is that the p21 mouse probe recognises the human p21 mRNA with less specificity than the mouse GAPDH probe recognises human GAPDH, hence the difference in the p21/GAPDH ratios observed between human cell and mouse cells. However, this piece of data must be taken cautiously since the appropriate human primary fibroblast cell line was not

available in the laboratory at the time of carrying out the experimental work. A proper comparison should be made in order to confirm this and the work is in progress in our laboratory at the moment.

Chapter 7

Conclusion

Since its cloning in 1986, the complete picture of the function of the ERCC1 gene and its encoded protein has remained a mystery. ERCC1 is known to play an essential role in nucleotide excision repair (NER) and a defect in ERCC1 function renders the cells hypersensitive to killing by UV-induced damage as well as damage induced by other chemical agents. In humans, defects in genes involved in NER result in a number of complex syndromes such as xeroderma pigmentosum (XP), Cockayne's syndrome (CS) and trichothiodystrophy (TTD), which share a number of clinical and cellular features, such as hypersensitivity to sunlight, slow growth, mental retardation, and impaired sexual development. In addition, there are several variants amongst XP, CS and TTD patients as well as combinations of XP and CS features (XP-CS) depending on which NER protein function is defective. Also, the incidence of features such as skin cancer susceptibility, neurological abnormalities and UV-sensitivity vary amongst the different syndromes and their variants.

The complexity of these syndromes has been shown to be due to the dual roles of some of the NER proteins, such as XPB and XPD, which appear to function as part of the cellular transcription machinery as well as the NER machinery. Thus, different defects in these proteins can affect their repair function only, resulting in XP, their transcription function only, resulting in repair-proficient TTD or both functions, resulting in repair-deficient TTD and the combined XP-CS. Defects in genes that are only involved in NER, such as XPA and XPC give rise to XP features only.

7.1 Why does the ERCC1 model differ from other XP mouse models?

Mouse models have helped to understand the genetic and biochemical basis behind these syndromes. For example, good XP-A (Nakane et al., 1995; deVries et al., 1995) and XP-C (Sands et al., 1995) mouse models that resemble most of the pathological changes shown by the human patients suffering from these types of XP have been developed. A knock out (k/o) mouse model for ERCC1 also exists (developed by McWhir *et al.* in 1993), but despite the initial expectations, the ERCC1 k/o mouse did not present any of the XP-related clinical features. This may not come as a complete surprise since ERCC1 was known to be unique in that, although its function is essential for NER, and indeed it corrects the UV-sensitive phenotype in one type of CHO mutants, it does not correct any of the XP complementation groups and there is no human syndrome associated with it. These facts led to the theory that an ERCC1-deficiency may be developmentally lethal, or alternatively, that ERCC1 may have additional functions (as happens with XPB and XPD) which, if defective, would result in a syndrome that does not resemble any of the NER-related pathologies. This function would be unique to ERCC1 and therefore, XPA or XPC mouse models would not show the additional ERCC1-related pathologies.

Support for this latter theory comes from studies that suggest that ERCC1 may be involved in recombination repair which has led to the proposition that the observed phenotype in the ERCC1 k/o mouse may be related to a defect in the recombination repair pathway as opposed to the NER pathway. Support for this theory comes from studies of the yeast ERCC1-XPF complex homolog, the Rad10-Rad1 complex in *S. cerevisiae*, the *S. pombe* homolog swi10 which have shown that these proteins do play an important role in recombinational repair and mating-type switching, respectively (Bardwell et al., 1994; Rodel et al., 1992). XPF has also been shown to have a homolog in *S. pombe*, rad16, and a homolog in *D. melanogaster* called mei-9 (Brookman et al., 1996), which has been shown to be required for meiotic recombination (Sekelski et al., 1995). Clues into the understanding of the ERCC1 function and its possible role in the pathology of disease may come from the ERCC1 k/o mouse model.

Mice deficient in ERCC1 show runting at birth, early death before weaning possibly due to hepatic failure, liver aneuploidy and polyploidy and elevated levels of p53 in liver, brain, and kidney (McWhir et al., 1993a). Interestingly, a k/o mouse model has been developed that is deficient in XPG (Harada et al., 1999), the 3' specific endonuclease that performs the dual incision with the XPF/ERCC1 5' endonuclease. XPG-deficient mice present a similar phenotype to the ERCC1 k/o mice in that they show retarded growth and die before weaning. Embryonic primary fibroblasts from these mice are sensitive to UV irradiation and are defective in NER and these cells show premature senescence. No ERCC1-like liver phenotype was apparent in the XPG null mice. Based on these findings XPG has also been proposed to have additional roles, such as repair of oxidative damage, outside NER (Harada et al., 1999). The work presented in this thesis attempts to show the mechanisms underlying the ERCC1-deficient phenotype.

7.2 The liver phenotype in ERCC1-deficient mice

From the studies presented in this thesis it is clear that the ERCC1-deficient phenotype is mainly due both to defects in the regulation of the cell cycle of the affected cells and defects in DNA damage-induced response mechanisms.

7.2.1 Liver morphology studies

Support for the first affirmation comes from morphology, immunohistochemistry and immunocytochemistry studies performed on livers from ERCC1 deficient animals along with ERCC1/p53 double null mice and wild type and p53-deficient mice as controls. It has been shown that in ERCC1-deficient livers the process of binucleation is inhibited and that this inhibition is bypassed in the absence of p53. Since binucleation is a process that requires consecutive rounds of replication, it is not surprising that p53 has a role in its regulation which coincides with its well characterised role in the control of the G1/S transition. We have also shown that ERCC1-deficient hepatocytes have elevated levels of the cyclin-dependent kinase inhibitor, p21, which is independent of their p53 status and that they are arrested mainly in G2. We propose that p21, in a p53-dependent manner, may be involved in the G2 arrest seen in the ERCC1-deficient hepatocytes and that it may also have a p53-independent role in the formation of

polyploidisation and premature ageing characteristics seen in ERCC1 null livers.

Support for the results discussed here comes from the study of another ERCC1-deficient mouse model (Weeda et al., 1997). Weeda and co-workers present morphological studies of hepatocyte nuclear size distribution in the liver of ERCC1-deficient and wild type animals that coincides with the data shown here. p21 levels in ERCC1-deficient animals are also reported to be elevated in their k/o mouse, although no data are shown. Weeda *et al.* also report that the primary fibroblasts derived from their ERCC1-deficient animals present characteristics typical of premature senescence in culture such as polyploidy. We have been consistently unable to see such behaviour in our primary fibroblast cultures. This difference is probably due to the different genetic background of the two mouse models used in these experiments.

7.2.2 UV irradiation studies

Support for the second affirmation comes from the study of the cell cycle control mechanisms elicited in ERCC1-deficient cells under conditions of exogenous DNA damage (UV irradiation). It is known that after UV mammalian cells inhibit DNA synthesis, albeit transiently, before proceeding with DNA replication. In human cells, if the DNA damage occurs while cells are replicating (S phase), this DNA synthesis inhibition is accompanied by a long delay in the entry of these cells into mitosis which allows the cells to repair the damage inflicted to their DNA. It is also known that this delay is significantly shorter in rodent cells (CHO cells reach mitosis 15 hours after UV and entry of human cells is delayed by 30 hours). From our studies it was clear that the UV-induced replication arrest shown by both the wild type and the ERCC1-deficient mouse and hamster cell lines was shorter than that shown by human cells defective in DNA repair (XP-A complementation group). This indicates that bypass of fork progression-arrest is NER independent in rodent cells, whereas there is a requirement for proficient DNA repair in order for human cells to recover from such arrest. However, and despite preliminary positive indications, it was also clear that there was no difference in the replication arrest kinetics seen in ERCC1- and ERCC2-deficient cell lines after UV, suggesting that there is no specificity for the requirement of ERCC1 in the UV-induced inhibition of DNA synthesis. In order to elucidate the mechanisms

that the cell uses to signal DNA damage and to see if ERCC1, as part of the NER machinery, is involved in this signalling process, we have studied the p53 and p21 response of ERCC1-deficient and wild type primary fibroblasts to UV-induced damage. Both p53 and p21 have been shown to be part of the cellular mechanisms to signal and prevent DNA damage from perpetuating, either through DNA replication (this is achieved via a p53/p21-induced G1/S arrest) or mitosis (this is achieved via a p53/p21-induced G2 arrest). We have shown that ERCC1-deficient mouse primary fibroblasts accumulate p53 protein at lower UV doses (5 J/m^2) than NER proficient primary fibroblasts (20 J/m^2) which is consistent with the fact that ERCC1-deficient fibroblasts cannot repair their DNA and therefore they accumulate DNA lesions at low UV doses. In terms of the p21 response to UV it has been shown that, after high UV fluences (50 J/m^2), the induction of p21 transcription is impaired in the ERCC1-deficient fibroblasts.

7.3 A model for the observed phenotype in ERCC1-deficient mice

Taking all the previous data into account, we propose a model to explain the phenotype observed in the ERCC1-deficient mice. In the absence of ERCC1, endogenous damage is accumulated in the liver which prompts the accumulation of p53 and p21. p53 and p21, in a p53-dependent manner, are likely to be involved in the control of the arrest that in hepatocytes occurs at the G2 checkpoint in the absence of proficient NER. p21 may also have an active role in a p53-independent manner in the regulation of the G2/M checkpoint control, promoting G2 arrest, and, more likely, controlling the hepatocyte differentiation and senescence processes. Both the p53-dependent and the p21-dependent mechanisms would have as an overall result the premature hepatic polyploidisation process seen in the ERCC1-deficient livers and their aged appearance.

7.3.1 Why ERCC1?

As mentioned before, other mouse models deficient in different proteins involved in NER exist (XPA, XPC and XPG), but none of them present the severe phenotype associated with the deficiency of ERCC1. There is extensive evidence that ERCC1 may be involved in recombinational processes both from studies of the ERCC1 protein

function and from analogy to ERCC1 homologues in other species (Rolig et al., 1995; Sargent et al., 1997; Bardwell et al., 1993; Bardwell et al., 1994; Rodel et al., 1992; Rodel et al., 1997; Ivanov and Haber, 1995). Following this line of thought it has already been proposed that the non-NER phenotype of the ERCC1-deficient mice is due to impairment of an additional ERCC1 function in another biological pathway, such as oxidative damage repair (McWhir et al., 1993a). Weeda *et al.* also propose that the reduced life span, the growth retardation and the liver, kidney and spleen manifestations they observe in ERCC1-deficient mice are most likely derived from impairment of the interstrand cross-link repair pathway. They also suggest that this is the basis for the features they observe in the ERCC1-mutant primary fibroblasts, i.e., enlarged polyploid nuclei and nuclear inclusions as well as replicative senescence (Weeda et al., 1997). As mentioned before, we fail to observe such a severe phenotype in kidney and spleen but this most likely due to the different life spans of the two models, and the fact that our mice die before such characteristics are developed. Another possibility is that the differences in the organ and primary fibroblasts phenotypes between the two models are due to the different genetic backgrounds of the mice used by the two laboratories.

7.3.2 Why liver?

Liver is an organ that is required for a large number of metabolic processes, such as lipid peroxidation, intermediate metabolism of carbohydrates and proteins, synthesis of proteins and detoxification and removal of foreign material such as bacteria, drugs and other noxious substances. All this results in the liver cells being exposed and producing large quantities of endogenous damaging agents such as oxygen-free radicals and toxic metabolic byproducts, such as malondialdehyde (MDA), a carbonyl compound which is the major by-product of arachidonic acid metabolism and lipid peroxidation (Weeda et al., 1997). MDA produces adducts and induces interstrand crosslinks which have been detected in liver DNA (Weeda et al., 1997).

If ERCC1, but none of the other NER genes, is involved in interstrand crosslink repair and/or oxidative damage repair it is possible that in its absence the liver accumulates endogenous DNA damage that is not properly repaired. As explained elsewhere in this

thesis (see Chapter 3, section 3.3) the liver responds to damage by becoming polyploid and this tendency to polyploidisation is very marked in mouse liver, compared to rat or human liver. Polyploidisation is also a marker of hepatocyte terminal differentiation. In the ERCC1-deficient liver the unrepaired damaged-DNA would elicit a damage response unique to the liver tissue that involves premature polyploidisation and terminal liver differentiation, probably through a p21-dependent pathway. This response to the existing unrepaired endogenous damage would explain the phenotype observed in these transgenic mice. It would be of interest to see what the outcome would be if p21 null mice were to be crossed with the ERCC1 null mice. It is possible, if the above theory is true, that the liver phenotype could be rescued in this way and the effects of an ERCC1 deficiency in other organs could be studied in the resulting longer-lived double null mice.

7.4 A role for ERCC1 in inhibition of replication after UV damage and premature ageing?

ERCC1 does not seem to play a role in the UV-induced DNA synthesis arrest seen in mammalian cells, although there is an indication that its absence affects the long-term induction of p21 after UV irradiation. Through these studies it has also been possible to verify the different response of rodent and human cells to UV-induced DNA damage and the consequences these differences may have in the way humans and rodents deal with deficiencies in the same genes.

Since the liver phenotype in ERCC1-deficient mice is reminiscent of premature ageing, the possibility that ERCC1 may be connected to the process of human accelerated ageing has also been investigated. ERCC1 RNA and protein levels as well as its genomic structure and cDNA sequence have been assessed in human primary fibroblasts derived from a patient suffering from Hutchinson-Gilford progeria (a rare human premature ageing syndrome). No evidence for mutations in the ERCC1 gene was found. However, the levels of ERCC1 mRNA and protein were found to be reduced in these fibroblasts compared to wild type transformed fibroblasts, but the reduction also affected other proteins involved in a variety of cellular functions indicating that the defect seen in these cells is probably of a complex origin. Furthermore, no detectable p53 or p21

response was detected in the progeria cells after UV irradiation, which opens up a number of interesting questions. These cells have already been shown to have a defect in the process of repair synthesis after UV, but no mechanism has been proposed to explain this phenomenon. It is possible that progeria cells are impaired in the detection of DNA damage and that therefore, DNA repair mechanisms are not efficiently elicited in these cells, resulting in accumulation of endogenous damage and premature senescence. This idea has also been tested by Weeda and collaborators who chose to study the possible involvement of ERCC1 in Roberts syndrome (Roberts syndrome patients display a variety of developmental abnormalities, including renal defects and malignancies and some patients exhibit, at the cellular level, nuclear abnormalities and UV sensitivity as well as cross-link sensitivity) but they failed to find defects in ERCC1 at the DNA and RNA level (Weeda et al., 1997). However, these two experiments do not rule out the possibility that ERCC1 mutations exist in other patients.

Overall, this thesis has brought together DNA repair, cell cycle regulation, responses to UV-induced and endogenous damage and cellular differentiation mechanisms to explain the liver phenotype observed in the ERCC1-deficient mice. This work has also opened the door for the study of a human premature ageing syndrome in the context of DNA repair and control of the cellular DNA damage-induced responses.

Bibliography

- Abdenur, J. E., Brown, W. T., Friedman, S., Smith, M., Zdanowicz, M., and Lifshitz, F. 1991. Endogenous growth-hormone resistance and malnutrition in children with Hutchinson-Gilford progeria syndrome (hgp). *Pediatric Research*, 29(4):A 73-A 73.
- Aboussekhra, A., Biggerstaff, M., Shivji, M., Vilpo, J., Moncollin, V., Podust, V., Protic, M., Hubscher, U., Egly, J., and Wood, R. 1995. Mammalian DNA nucleotide excision-repair reconstituted with purified protein-components. *Cell*, 80(6):859-868.
- Abrahams, P., Schouten, R., Vanlaar, T., Houweling, A., Terleth, C., and Vandereb, A. 1995. Different regulation of p53 stability in UV-irradiated normal and DNA-repair deficient human-cells. *Mutation Research-DNA Repair*, 336:169-180.
- Atadja, P., Wong, H., Garkavtsev, I., Veillette, C., and Riabowol, K. 1995. Increased activity of p53 in senescing fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America*, 92(18):8348-8352.
- Bardwell, A. J., Bardwell, L., Johnson, D. K., and Friedberg, E. C. 1993. Yeast DNA recombination and repair proteins rad1 and rad10 constitute a complex in vivo mediated by localized hydrophobic domains. *Molecular Microbiology*, 8(6):1177-1188.
- Bardwell, A. J., Bardwell, L., Tomkinson, A. E., and Friedberg, E. C. 1994. Specific cleavage of model recombination and repair intermediates by the yeast rad1-rad10 DNA endonuclease. *Science*, 265(5181):2082-2085.
- Basi, G. and Draetta, G. 1995. The cdc2 kinase: structure, activation and its role at mitosis in vertebrate cells. In (Hutchison and Glover, 1995), pages 106-143.
- Bates, S., Ryan, K. M., Phillips, C., and Vousden, K. 1998. Cell cycle arrest and DNA endoreduplication following p21^{Waf1/Cip1} expression. *Oncogene*, 17:1691-1703.
- Bates, S. and Vousden, K. 1996. p53 in signaling checkpoint arrest or apoptosis. *Current Opinion in Genetics and Development*, 6(1):1-7.
- Baynton, K., Bresson-Roy, A., and Fuchs, R. P. P. 1998. Analysis of damage tolerance pathways in *saccharomyces cerevisiae*: a requirement for rev3 DNA polymerase in translesion synthesis. *Molecular and Cellular Biology*, 18(2):960-966.
- Behrens, B., Hamilton, T., Masuda, H., Grotzinger, K., Whang-Peng, J., Louie, K., Knutsen, T., McKoy, W., Young, R., and Ozols, R. 1987. Characterization of a cis-diamminedichloroplatinum(ii)-resistant human ovarian cancer cell line and its use in evaluation of platinum analogues. *Cancer Research*, 47(2):414-418.
- Berger, C. and Edenberg, H. 1986. Pyrimidine dimers block simian virus 40 replication forks. *Molecular and Cell Biology*, 6:3443-3450.
- Bessho, T., Sancar, A., Thompson, L. H., and Thelen, M. P. 1997. Reconstitution of human excision nuclease with recombinant XPF-ERCC1 complex. *Journal of Biological Chemistry*, 272(6):3833-3837.

- Bierne, H. and Michel, B. 1994. When replication forks stop. *Molecular Microbiology*, 13(1):17-23.
- Biggerstaff, M., Szymkowski, D. E., and Wood, R. D. 1993. Co-correction of the ERCC1, ERCC4 and xeroderma-pigmentosum group-f DNA-repair defects in-vitro. *EMBO Journal*, 12(9):3685-3692.
- Blow, J. 1993. Preventing re-replication of DNA in a single cell cycle: evidence for a Replication Licensing Factor. *Journal of Cell Biology*, 122:993.
- Blow, J. 1995. S phase and its regulation. In (Hutchison and Glover, 1995), pages 177-205.
- Bohm, N. and Noltemeyer, N. 1981. Development of binuclearity and DNA-polyploidisation in the growing mouse liver. *Histochemistry*, 72:55-61.
- Bond, J., Wyllie, F., and Wynfordthomas, D. 1994. Escape from senescence in human-diploid fibroblasts induced directly by mutant p53. *Oncogene*, 9:1885-1889.
- Bond, J. A., Blaydes, J. P., Rowson, J., Haughton, M. F., Smith, J. R., Wynfordthomas, D., and Wyllie, F. S. 1995. Mutant p53 rescues human-diploid cells from senescence without inhibiting the induction of Sdi1/Waf1. *Cancer Research*, 55(11):2404-2409.
- Bootsma, D. and Hoeijmakers, J. 1994. The molecular basis of nucleotide excision repair syndromes. *Mutation Research*, 307:15.
- Boulikas, T. 1996. Xeroderma-pigmentosum and molecular-cloning of DNA-repair genes. *Anticancer Research*, 16(2):693-708.
- Brasch, K. 1980. Endopolyploidy in vertebrate liver: An evolutionary perspective. *Cell Biology International Reports*, 4(2):217-226.
- Brookman, K. W., Lamerdin, J. E., Thelen, M. P., Hwang, M., Reardon, J. T., Sancar, A., Zhou, Z. Q., Walter, C. A., Parris, C. N., and Thompson, L. H. 1996. ERCC4 (XPF) encodes a human nucleotide excision-repair protein with eukaryotic recombination homologs. *Molecular and Cellular Biology*, 16(11):6553-6562.
- Brown, J. P., Wei, W. Y., and Sedivy, J. M. 1997. Bypass of senescence after disruption of p21(Cip1/Waf1) gene in normal diploid human fibroblasts. *Science*, 277(5327):831-834.
- Brown, W. T. 1992. Progeria: A human-disease model of accelerated ageing. *The American Journal of Clinical Nutrition*, 55:1222S-1224S.
- Brugal, G. 1992. HOME: Highly optimised microscope environment. *Cytometry*, 13:109-116.
- Brugarolas, J., Chandrasekaran, C., Gordon, J. I., Beach, D., Jacks, T., and Hannon, G. J. 1995. Radiation-induced cell-cycle arrest compromised by p21 deficiency. *Nature*, 377(6549):552-557.
- Burki, H., Lam, R., and Wood, R. 1980. UV-light-induced mutations in synchronous CHO cells. *Mutation Research*, 69:347-356.
- Carr, A., Schmidt, H., Kirchhoff, S., Muriel, W., Sheldrick, K., Griffiths, D., Basmacioghi, C., Subramani, S., Clegg, M., and Nasim, A. 1994. The rad16 gene of *Schizosaccharomyces pombe*: a homolog of the rad1 gene of *Saccharomyces cerevisiae*. *Molecular and Cellular Biology*, 14:2029-2040.
- Carty, M., Zernik-Kobak, M., McGrath, S., and Dixon, K. 1994. UV light-induced DNA-synthesis arrest in HeLa-cells is associated with changes in phosphorylation of human single-stranded DNA-binding protein. *EMBO Journal*, 13:2114-2123.
- Cayrol, C. and Ducommun, B. 1997. Interaction of the cyclin-dependent kinase inhibitor p21 with PCNA: A link between cell cycle and DNA repair. *M S-Medicine*

- Sciences*, 13(11):1259–1265.
- Chen, J., Chen, S., Saha, P., and Dutta, A. 1995. Separate domains of p21 involved in the inhibition of cdk kinase and pcna. *Nature*, 374:386–388.
- Chen, J., Chen, S., Saha, P., and Dutta, A. 1996a. P21(Cip1/Waf1) disrupts the recruitment of human fen1 by proliferating-cell nuclear antigen into the DNA-replication complex. *Proceedings Of The National Academy Of Sciences Of The United States Of America*, 93:11597–11602.
- Chen, J., Peters, R., Saha, P., Lee, P., Theodoras, A., Pagano, M., Wagner, G., and Dutta, A. 1996b. A 39-amino-acid fragment of the cell-cycle regulator p21 is sufficient to bind PCNA and partially inhibit DNA-replication in-vivo. *Nucleic Acids Research*, 24:1727–1733.
- Chin, P. L., Momand, J., and Pfeifer, G. P. 1997. In vivo evidence for binding of p53 to consensus binding sites in the p21 and Gadd45 genes in response to ionizing radiation. *Oncogene*, 15(1):87–99.
- Chomczynski, P. and Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate phenol chloroform extraction. *Analytical Biochemistry*, 162(1):156–159.
- Clarkson, J. and Hewitt, R. 1976. Significance of dimers to the size of newly synthesised DNA in UV-irradiated chinese hamster ovary cells. *Biophysics Journal*, 16:1155–1164.
- Cleaver, J. 1965. Investigations into the effects of ultraviolet light on the rate of deoxyribonucleic acid synthesis in mammalian cells. *Biochimica Et Biophysica Acta*, 108:42–52.
- Cleaver, J. 1968. Defective repair replication of DNA in xeroderma pigmentosum. *Nature, London*, 218:652.
- Cleaver, J., Kaufmann, W., Kapp, L., and Park, S. 1983. Replicon size and excision repair as factors in the inhibition and recovery of DNA synthesis from ultraviolet damage. *Biochimica Et Biophysica Acta*, 739:207–215.
- Coleman, T. and Dunphy, W. 1994. Cdc2 regulatory factors. *Current Opinion in Cell Biology*, 6:877–882.
- Colige, A., Nusgens, B., and Lapiere, C. M. 1991a. Altered response of progeria fibroblasts to epidermal growth-factor. *Journal of Cell Science*, 100(Pt3):649–655.
- Colige, A., Roujeau, J. C., Delarocque, F., Nusgens, B., and Lapiere, C. M. 1991b. Abnormal gene-expression in skin fibroblasts from a hutchinson- gilford patient. *Laboratory Investigation*, 64(6):799–806.
- Cordeiro-Stone, M., Schumacher, R., and Meneghini, R. 1979. Structure of replication fork in UV light irradiated human cells. *Biophysics Journal*, 27:287–300.
- Cordonnier, A. M., Lehmann, A. R., and Fuchs, R. P. P. 1999. Impaired translesion synthesis in xeroderma pigmentosum variant extracts. *Molecular and Cellular Biology*, 19(3):2206–2211.
- Cox, L. S. 1997. Multiple pathways control cell growth and transformation: Overlapping and independent activities of p53 and p21(Cip1/Waf1/Sdi1). *Journal of Pathology*, 183(2):134–140.
- Dahle, D., Griffiths, T., and Carpenter, J. 1980. Inhibition and recovery of DNA synthesis in UV irradiated chinese hamster V-79 cells. *Photochemistry & Photobiology*, 32:157–165.
- Davis, I. 1999. Visualising fluorescence in drosophila - optical detection in thick specimens. In Allan, V., editor, *Visualising proteins by fluorescence, a practical approach*.

- Book in preparation.
- de Boer, J., de Wit, J., van Steeg, H., Berg, R., Morreau, H., Visser, P., Lehmann, A., Duran, M., Hoeijmakers, J., and Weeda, G. 1998. A mouse model for the basal transcription/ repair syndrome trichothiodystrophy. *Molecular Cell*, 1:981-990.
- deLaat, W. L., Appeldoorn, E., Jaspers, N. G. J., and Hoeijmakers, J. H. J. 1998. DNA structural elements required for ERCC1-XPF endonuclease activity. *Journal of Biological Chemistry*, 273(14):7835-7842.
- Deng, C. X., Zhang, P. M., Harper, J. W., Elledge, S. J., and Leder, P. 1995. Mice lacking p21(c/p1/Waf1) undergo normal development, but are defective in G1 checkpoint control. *Cell*, 82(4):675-684.
- Denko, N., Giaccia, A., Peters, B., and Stamato, T. D. 1989. An asymmetric field inversion gel-electrophoresis method for the separation of large DNA-molecules. *Analytical Biochemistry*, 178(1):172-176.
- DePamphilis, M., editor 1996. *DNA Replication in Eukaryotic Cells*. Cold Spring Harbor Laboratory Press, New York.
- deVries, A., Vanoostrom, C. T. M., Hofhuis, F. M. A., Dortant, P. M., Berg, R. J. W., Degruyl, F. R., Wester, P. W., Vankreijl, C. F., Capel, P. J. A., Vansteeg, H., and Verbeek, S. J. 1995. Increased susceptibility to ultraviolet-b and carcinogens of mice lacking the DNA excision-repair gene XPA. *Nature*, 377(6545):169-173.
- Doniger, J. 1978. DNA replication in ultraviolet light irradiated chinese hamster cells: The nature of replicon inhibition and post-replication repair. *Journal of Molecular Biology*, 120:433-446.
- Drapkin, R., Reardon, J., Ansari, A., Huang, J.-C., Zawel, L., Ahn, K., Sancar, A., and Reinberg, D. 1994. Dual role of TFIIH in DNA excision repair and in transcription by RNA polymeraseII. *Nature, London*, 368:769-772.
- Dumaz, N., Drougard, C., Sarasin, A., and DayaGrosjean, L. 1998. Recovery of the normal p53 response after UV-treatment in DNA repair deficient fibroblasts by retroviral-mediated correction with the XPD gene. *Carcinogenesis*, In Press.
- Dyer, C. A. E. and Sinclair, A. J. 1998. The premature ageing syndromes: insights into the ageing process. *Age and Ageing*, 27(1):73-80.
- Earnshaw, W. C. and Rothfield, N. 1985. Identification of a family of human centromere proteins using autoimmune sera from patients with scleroderma. *Chromosoma (Berl)*, 91(3-4):313-321.
- Edenberg, H. 1976. Inhibition of DNA replication by ultra-violet light. *Biophysics Journal*, 16:849-860.
- El-Deiry, W., Harper, J., O'Connor, P., Velculescu, V., Canman, C., Jackman, J., Pietenpol, J., Burrell, M., Hill, D., Wang, Y., and Vogelstein, B. 1994. WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Research*, 54:1169-1174.
- El-Deiry, W., Tokino, T., Velculescu, V., Levy, D., Parsons, R., Trent, J., Lin, D., Mercer, W., Kinzler, K., and Vogelstein, B. 1993. WAF1, a potential mediator of p53 tumor suppression. *Cell*, 75:817-825.
- Elbendary, A., Berchuck, A., Davis, P., Havrilesky, L., Bast, R., Iglehart, J., and Marks, J. 1994. Transforming growth-factor-beta-1 can induce CIP1/WAF1 expression independent of the p53 pathway in ovarian-cancer cells. *Cell Growth and Differentiation*, 5(12):1301-1307.
- Elledge, S. and Harper, J. 1994. Cdk inhibitors - on the threshold of checkpoints and development. *Current Opinion In Cell Biology*, 6:847-852.

- Ellis, N., J., G., and T.Z, Y. 1995. The bloom's syndrome gene product is homologous to RecQ helicases. *Cell*, 83:655–656.
- Epstein, C. 1967a. Cell size, nuclear content, and the development of polyploidy in the mammalian liver. *Proceedings Of The National Academy Of Sciences Of The United States Of America*, 57:327–334.
- Epstein, C. 1967b. Nuclear ploidy in mammalian parenchymal liver cells. *Nature*, 124:1050–1051.
- Evans, E., Moggs, J. G., Hwang, J. R., Egly, J. M., and Wood, R. D. 1997. Mechanism of open complex and dual incision formation by human nucleotide excision repair factors. *EMBO Journal*, 16(21):6559–6573.
- Fan, S., Smith, M., Rivet, D., Zhan, Q., Kohn, W., Fornace, A., and O'Connor, P. 1995. Disruption of p53 sensitizes breast cancer mcf-7 cells to cisplatin and pentoxifylline. *Cancer Research*, 55:1649–1654.
- Feinberg, A. P. and Vogelstein, B. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry*, 132(1):6–13.
- Freeman, S. E., Blackett, A. D., Monteleone, D. C., Setlow, R. B., Sutherland, B. M., and Sutherland, J. C. 1986. Quantitation of radiation-induced, chemical-induced, or enzyme-induced single-strand breaks in nonradioactive DNA by alkaline gel-electrophoresis - application to pyrimidine dimers. *Analytical Biochemistry*, 158(1):119–129.
- Friedberg, E., Walker, G., and Siede, W., editors 1995. *DNA repair and mutagenesis*. American Society for Microbiology, Washington.
- Friedberg, E. and Wood, R. 1996. DNA excision repair pathways. In (DePamphilis, 1996), pages 249–269.
- Gadbois, D. M. and Lehnert, B. E. 1997. Cell cycle response to DNA damage differs in bronchial epithelial cells and lung fibroblasts. *Cancer Research*, 57(15):3174–3179.
- Ganesan, A. and Seawell, P. 1975. The effect of *lex a* and *rec f* mutations on post-replication repair and synthesis in *E. coli* k12. *Molecular & General Genetics*, 141:189–205.
- Gary, R., Ludwig, D., Cornelius, H., MacInnes, M., and Park, M. 1997. The DNA repair endonuclease XPG binds to proliferating cell nuclear antigen (PCNA) and shares sequence elements with the PCNA-binding regions of FEN-1 and cyclin-dependent kinase inhibitor p21. *The Journal of Biological Chemistry*, 272:24522–24529.
- Gatti, R. and Swift, M., editors 1985. *Ataxia telangiectasia, Genetics, Neuropathology and Immunology of a Degenerative Disease of Childhood*. Liss, New York.
- Gibbs, P., McGregor, W., Maher, V., Nisson, P., and Lawrence, C. 1998. A human homolog of the *Saccharomyces cerevisiae* rev3 gene, which encodes the catalytic subunit of DNA polymerase ζ . *Proceedings of the National Academy of Sciences of the United States of America*, 95:6876–6880.
- Giro, M. and Davidson, J. M. 1993. Familial co-segregation of the elastin phenotype in skin fibroblasts from Hutchinson-Gilford progeria. *Mechanisms of Ageing and Development*, 70(3):163–176.
- Graña, X. and Reddy, E. 1995. Cell cycle control in mammalian cell: role of cyclins, cyclin dependent kinases (cdks), growth suppressor genes and cyclin-dependent kinase inhibitors (ckis). *Oncogene*, 11:211–219.
- Grawunder, U., Wilm, M., Wu, X., Kulesza, P., Wilson, T., Mann, M., and Lieber, M. 1997. Activity of DNA ligase IV stimulated by complex formation with XRCC4

- protein in mammalian cells. *Nature*, 388(31):492-495.
- Griffiths, T. and Ling, S. 1987. Activation of alternative sites of replicon initiation in chinese hamster cells exposed to ultraviolet light. *Mutation Research*, 184:39-46.
- Griffiths, T. and Ling, S. 1989. Effects of UV light on DNA chain growth and replicon initiation in human cells. *Mutation Research*, 218:87-94.
- Griffiths, T., Taft, S., and Ling, S. 1990. Effect of UV light on DNA replication and chain elongation in chinese hamster UV61 cells. *Mutation Research*, 236:51-58.
- Guadagno, T. and Newport, J. 1996. Cdk2 kinase is required for entry into mitosis as a positive regulator of cdc2-cyclin B kinase activity. *Cell*, 84:73-82.
- Guzder, S. N., Sung, P., Prakash, L., and Prakash, S. 1998. Affinity of yeast nucleotide excision repair factor 2, consisting of the rad4 and rad23 proteins, for ultraviolet damaged DNA. *Journal of Biological Chemistry*, 273(47):31541-31546.
- Haapajarvi, T., Pitkanen, K., Tsubari, M., and Laiho, M. 1997. p53 transactivation and protein accumulation are independently regulated by UV light in different phases of the cell cycle. *Molecular and Cellular Biology*, 17(6):3074-3080.
- Habraken, Y., Sung, P., Prackash, L., and Prakash, S. 1993. Yeast excision repair gene RAD2 encodes a single stranded DNA endonuclease. *Nature*, 366:365.
- Hanawalt, P. 1994. Transcription-coupled repair and human-disease. *Science*, 266:1957-1958.
- Hanawalt, P. 1995. DNA-repair comes of age. *Mutation Research-DNA Repair*, 336:101-113.
- Hanawalt, P. and Painter, R. 1985. On the nature of a 'DNA processing' defect in ataxia-telangiectasia. In (Gatti and Swift, 1985), pages 67-71.
- Harada, Y.-N., Shiomi, N., Koiko, M., Ikawa, M., Okabe, M., Hirota, S., Kitamura, Y., Kitagawa, M., Matsunaga, T., Nikaido, O., and Shiomi, T. 1999. Postnatal growth failure, short life span, and early onset of cellular senescence and subsequent immortalization in mice lacking xeroderma pigmentosum group G gene. *Molecular and Cellular Biology*, 19(3):2366-2372.
- Hartwell, L. and Kastan, M. 1994. Cell cycle control and cancer. *Science*, 266:1821-1828.
- Harvey, M., Sands, A. T., Weiss, R. S., Hegi, M. E., Wiseman, R. W., Pantazis, P., Giovanella, B. C., Tainsky, M. A., Bradley, A., and Donehower, L. A. 1993. In-vitro growth-characteristics of embryo fibroblasts isolated from p53-deficient mice. *Oncogene*, 8(9):2457-2467.
- Haseltine, W., Gordon, L., Lindon, C., Grafstrom, R., Shaper, N., and Grossmann, L. 1980. Cleavage of pyrimidine dimers in specific DNA sequences by a pyrimidine dimer DNA-glycosylase of *M. luteus*. *Nature, Lond.*, 285:634-641.
- Hayashi, T., Takao, M., Tanaka, K., and Yasui, A. 1998. ERCC1 mutations in UV-sensitive chinese hamster ovary (CHO) cell lines. *Mutation Research-DNA Repair*, 407(3):269-276.
- Hayles, J., Fisher, D., Wollard, A., and Nurse, P. 1994. Temporal order of s phase and mitosis in fission yeast is determined by the state of the p34-cdc2-mitotic b cyclin complex. *Cell*, 78:813-822.
- He, Z. G. and Ingles, C. J. 1997. Isolation of human complexes proficient in nucleotide excision repair. *Nucleic Acids Research*, 25(6):1136-1141.
- Humbert, S., van Vuuren, H., Lutz, Y., Hoeijmakers, J., Egly, J.-M., and Moncollin, V. 1994. Characterisation of p44/1 and p34 subunits of the BTF2(TFIIH) transcription/repair factor. *EMBO Journal*, 13:2393-2398.

- Huschtscha, L. and Holliday, R. 1983. Limited and unlimited growth of SV40-transformed cells from human diploid MRC-5 fibroblasts. *Journal of Cell Science*, 63:77-99.
- Hutchison, C. and Glover, D., editors 1995. *Cell cycle control*. IRL Press at Oxford University Press, New York.
- Iliakis, G. E., Cicilioni, O., and Metzger, L. 1991. Measurement of DNA double-strand breaks in CHO cells at various stages of the cell-cycle using pulsed field gel-electrophoresis - calibration by means of ¹²⁵I decay. *International Journal of Radiation Biology*, 59(2):343-357.
- Isaacs, J. S., Chiao, C., Merrick, B. A., Selkirk, J. K., Barrett, J. C., and Weissman, B. E. 1997. p53-dependent p21 induction following gamma-irradiation without concomitant p53 induction in a human peripheral neuroepithelioma cell line. *Cancer Research*, 57(14):2986-2992.
- Ivanov, E. and Haber, J. 1995. RAD1 and RAD10, but not other excision repair genes, are required for double-strand break-induced recombination in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology*, 15:2245-2251.
- Iwanejko, L. and Jones, N. 1998. Recent advances in DNA repair and recombination. *Mutation Research*, 408:227-236.
- Jackson, P., Chevalier, S., Philippe, M., and Kirschner, M. 1995. Early events in DNA-replication require cyclin-E and are blocked by p21(Cip1). *Journal Of Cell Biology*, 130:755-769.
- Jansen, L. E. T., Verhage, R. A., and Brouwer, J. A. 1998. Preferential binding of yeast rad4.rad23 complex to damaged DNA. *Journal of Biological Chemistry*, 273(50):33111-33114.
- Janson, W., Brandner, G., and Siegel, J. 1997. Butyrate modulates DNA-damage-induced p53 response by induction of p53-independent differentiation and apoptosis. *Oncogene*, 15(12):1395-1406.
- Jeggo, P., Carr, A., and Lehmann, A. 1998. Splitting the : distinct repair and checkpoint defects in ataxia-telangiectasia. *Trends in Genetics*, 14(8):312-316.
- Jiang, H., Lin, J., Su, Z., Collart, F., Huberman, E., and Fisher, P. 1994. Induction of differentiation in human promyelocytic HL-60 leukemia-cells activates p21, Waf1/Cip1, expression in the absence of p53. *Oncogene*, 9(11):3397-3406.
- Johnson, M., Dimitrov, D., Vojta, P. J., Barrett, J. C., Noda, A., Pereiras-Smith, O. M., and Smith, J. R. 1994. Evidence for a p53-independent pathway for up-regulation of Sdi1/Cip1/Waf1/p21 RNA in human-cells. *Molecular Carcinogenesis*, 11(2):59-64.
- Jonsson, Z. O., Hindges, R., and Hubscher, U. 1998. Regulation of DNA replication and repair proteins through interaction with the front side of proliferating cell nuclear antigen. *EMBO Journal*, 17(8):2412-2425.
- Kantor, G., Barsalou, L., and Hanawalt, P. 1990. Selective repair of specific chromatin domains in UV-irradiated cells from xeroderma pigmentosum complementation group C. *Mutation research*, 235:171-180.
- Kaufmann, W. and Cleaver, J. 1981. Mechanisms of inhibition of DNA replication by ultraviolet light in normal human and xeroderma pigmentosum fibroblasts. *Journal of Molecular Biology*, 149:171-187.
- Kaufmann, W., Cleaver, J., and Painter, R. 1980. Ultraviolet radiation inhibits replicon initiation in S phase human cells. *Biochimica Et Biophysica Acta*, 608:191-195.
- Kaufmann, W. and Paules, R. 1996. DNA-damage and cell-cycle checkpoints. *Faseb Journal*, 10:238-247.

- Kearsey, S., Labib, K., and Maiorano, D. 1996. Cell cycle control of eukaryotic DNA replication. *Current Opinion in Genetics & Development*, 6:208–214.
- Khan, Q. A., Vousden, K. H., and Dipple, A. 1997. Cellular response to DNA damage from a potent carcinogen involves stabilization of p53 without induction of p21(Waf1/Cip1). *Carcinogenesis*, 18(12):2313–2318.
- Kirkwood, T. 1989. DNA, mutations and ageing. *Mutation Research*, 219:1–7.
- Klimek, M. and Vlasinova, M. 1966. Thymine and uracyl-thymine dimers and deoxyribonucleic acid synthesis in mammalian cells irradiated with ultraviolet light. *International Journal of Radiation Biology*, 11(4):329–337.
- Kraemer, K., D.D., L., Parris, C., Gozukara, E., Moriwaki, S., Adelberg, S., and Seidman, M. 1994. Xeroderma pigmentosum and related disorders: examining the linkage between defective DNA repair and cancer. *Journal of Investion in Dermatology*, 103:965.
- Kraemer, K., Lee, M., and Scotto, J. 1987. Xeroderma pigmentosum: cutaneous, ocular and neurological abnormalities in 830 published cases. *Archives of Dermatology*, 123:241.
- Kriwacki, B., Hengst, L., Tennant, L., Reed, S., and Wright, P. 1996. Structural studies of p21(Waf1/Cip1/Sdi1) in the free and cdk2- bound state - conformational disorder mediates binding diversity. *Proceedings Of The National Academy Of Sciences Of The United States Of America*, 93:11504–11509.
- Kudryavtsev, R., Hengst, L., Tennant, L., Reed, S., and Wright, P. 1993. Human hepatocyte polyploidization in the course of life cycle. *Virchows Archives of B Cell Pathology Incl. molecular Pathology*, 64:387–393.
- Kuwano, K., Hagimoto, N., Nomoto, Y., Kawasaki, M., Kunitake, R., Fujita, M., Miyazaki, H., and Hara, N. 1997. p53 and p21 (Waf1/Cip1) mRNA expression associated with DNA damage and repair in acute immune complex alveolitis in mice. *Laboratory Investigation*, 76(2):161–169.
- Lees, E. and Harlow, E. 1995. Cancer and the cell cycle. In (Hutchison and Glover, 1995), pages 228–263.
- Lehmann, A. 1972. Postreplication repair of DNA in ultra-violet-irradiated mammalian cells. *Journal of Molecular Biology*, 66:319–337.
- Lehmann, A. 1979. The relationship between pyrimidine dimers and replicating DNA in UV-irradiated human fibroblasts. *Nucleic Acids Research*, 7:1901–1911.
- Lehmann, A. 1981. DNA replication in mammalian cells damaged by mutagens. In Seeberg, E. and Kleppe, K., editors, *Chromosome damage and repair*, pages 383–388. Plenum Publishing Corporation, New York.
- Lehmann, A., Kirk-Bell, S., Arlett, C., Paterson, M., Lohman, P., de Weerd-Kastelein, H., and Bootsma, D. 1975. Xeroderma pigmentosum cells with normal levels of excision repair have a defect in DNA synthesis after UV-irradiation. *Proceedings of the National Academy of Sciences of the United States of America*, 72:219–223.
- Lehmann, A., Kirk-Bell, S., and Mayne, L. 1979. Abnormal kinetics of DNA synthesis in UV-irradiated cells from patients with cockayne's syndrome. *Cancer Research*, 39:4237–4241.
- Li, L., Elledge, S. J., Peterson, C. A., Bales, E. S., and Legerski, R. J. 1994a. Specific association between the human DNA-repair proteins XPA and ERCC1. *Proceedings of the National Academy of Sciences of the United States of America*, 91(11):5012–5016.
- Li, L., Peterson, C., Lu, X., and Legerski, R. 1995. Mutations in XPA that prevent

- association with ERCC1 are defective in nucleotide excision-repair. *Molecular And Cellular Biology*, 15(4):1993–1998.
- Li, R., Hannon, G., Beach, D., and Stillman, B. 1996. Subcellular-distribution of p21 and PCNA in normal and repair- deficient cells following DNA-damage. *Current Biology*, 6:189–199.
- Li, R., Waga, S., Hannon, G., Beach, D., and Stillman, B. 1994b. Differential-effects by the p21 cdk inhibitor on PCNA-dependent DNA-replication and repair. *Nature*, 371:534–537.
- Lin, J., Reichner, C., Wu, X., and Levine, A. 1996. Analysis of wild-type and mutant p21(WAF-1) gene activities. *Molecular And Cellular Biology*, 16:1786–1793.
- Lin, W., Wu, X., and Wang, Z. 1999. A full length cDNA of hREV3 is predicted to encode DNA polymerase ζ for damage-induced mutagenesis in humans. *Mutation Research-DNA repair*, 433:89–98.
- Lindahl, T. and Barnes, D. 1992. Mammalian DNA ligases. *Annual Reviews of Biochemistry*, 61:251–281.
- Lipman, J. M., Applegatestevens, A., Soyka, L. A., and Hart, R. W. 1989. Cell-cycle defect of DNA-repair in progeria skin fibroblasts. *Mutation Research*, 219(5-6):273–281.
- Lu, X. and Lane, D. 1993. Differential induction of transcriptionally active p53 following UV or ionizing radiation: Defects in chromosome instability syndromes? *Cell*, 75:765–778.
- Maher, V., Dorney, D., Mendrala, A., Konze-Thomas, B., and McCormick, J. 1979. DNA excision repair processes in human cells eliminate the cytotoxic and mutagenic consequences of UV irradiation. *Mutation Research*, 62:311–323.
- Maher, V., Ouellete, L., Curren, R., and McCormick, J. 1976. Frequency of UV light-induced mutations is higher in xeroderma pigmentosum variant cells than in normal human cells. *Nature Lond.*, 261:593–595.
- Maltzman, W. and Czyzyk, L. 1984. UV irradiation stimulates levels of p53 cellular tumor antigen in nontransformed mouse cells. *Molecular And Cellular Biology*, 4(9):1689–1694.
- Maquart, F. X., Bellon, G., Gillery, P., Borel, J. P., Labeille, B., Risbourg, B., and Denoeux, J. P. 1988. Increased secretion of fibronectin and collagen by progeria (Hutchinson-Gilford) fibroblasts. *European Journal of Pediatrics*, 147(4):442–442.
- Marinoni, J.-C., Roy, R., Vermeulen, W., Miniou, P., Lutz, Y., Weeda, G., Seroz, T., Molina-Gomez, D., Hoeijmakers, J., and Egly, J.-M. 1997. Cloning and characterisation of p52, the fifth subunit of the core of the transcription/DNA repair factor TFIIH. *The EMBO Journal*, 16(5):1093–1102.
- Matsunaga, T., Mu, D., Park, C. H., Reardon, J. T., and Sancar, A. 1995. Human DNA-repair excision nuclease - analysis of the roles of the subunits involved in dual incisions by using anti-XPG and anti-ERCC1 antibodies. *Journal of Biological Chemistry*, 270(35):20862–20869.
- Matsunaga, T., Park, C. H., Bessho, T., Mu, D., and Sancar, A. 1996. Replication protein-a confers structure-specific endonuclease activities to the XPF-ERCC1 and XPG subunits of human DNA-repair excision nucleases. *Journal of Biological Chemistry*, 271(19):11047–11050.
- McDonald, E., Wu, G., Waldman, T., and El-Deiry, W. 1996. Repair defect in p21(Waf1/Cip1)-/- human cancer-cells. *Cancer Research*, 56:2250–2255.
- McWhir, J., Selfridge, J., Harrison, D., Squires, S., and Melton, D. 1993a. Mice with

- DNA-repair gene (ERCC-1) deficiency have elevated levels of p53, liver nuclear abnormalities and die before weaning. *Nature Genetics*, 5:217–224.
- McWhir, J., Wu, C., and Melton, D. 1993b. A combined genetic and biochemical mouse model for Lesch-Nyhan syndrome. *Journal Of Cellular Biochemistry*, page 244.
- Medvedev, Z. 1986. Age-related polyploidisation of hepatocytes: The cause and possible role. *Experimental Gerontology*, 21:277–282.
- Melton, D. W., Ketchen, A. M., Nunez, F., Bonatti-Abbondandolo, S., Abbondandolo, A., Squires, S., and Johnson, R. T. 1998. Cells from ERCC1-deficient mice show increased genome instability and a reduced frequency of S-phase-dependent illegitimate chromosome exchange but a normal frequency of homologous recombination. *Journal of Cell Science*, 111(3):395–404.
- Meneghini, R. 1976. Gaps in DNA synthesised by ultraviolet light-irradiated W138 human cells. *Biochimica Et Biophysica Acta*, 425:419–427.
- Meneghini, R. and de Mello Filho, A. 1983. Rate of DNA synthesis in mammalian cells irradiated with ultraviolet light: A model based on the variations in the rate of movement of the replication fork and in the number of active replicons. *Journal of Theoretical Biology*, 100:359–372.
- Meneghini, R. and Menck, C. 1978. Pyrimidine dimers in DNA strands of mammalian cells synthesised after UV-irradiation. In Hanwalt, P.C. and Friedberg, E. and Fox, C., editors, *DNA Repair Mechanisms*, pages 493–497. New York: Academic Press, New York.
- Meneghini, R., Menck, C., and Schumacher, R. 1981. Mechanism of tolerance to DNA lesions in mammalian cells. *Quarterly Reviews of Biophysics*, 14:381–432.
- Merlo, G. R., Fiore, L., Basolo, F., WoodsCook, K., and Hynes, N. E. 1997. In mammary epithelial cells p53-mediated apoptosis in response to DNA damage is dependent on the agent and can be influenced by growth factors. *Endocrine-Related Cancer*, 4(1):55–66.
- Meyn, M. 1995. Ataxia-telangiectasia and cellular responses to DNA damage. *Cancer Research*, 55:5991–6001.
- Meyn, R., Kasschau, M., and Hewitt, R. 1977. The recovery of normal DNA replication kinetics in UV-irradiated chinese hamster cells. *Mutation Research*, 44:129–138.
- Meyn, R., Vizard, D., Hewitt, R., and Humphrey, R. 1974. The fate of pyrimidine dimers in the DNA of UV-irradiated chinese hamster cells. *Photochemistry & Photobiology*, 55:5991–6001.
- Michieli, P., Chedid, M., Lin, D., Pierce, J., Mercer, W., and Givol, D. 1994. Induction of Waf1/Cip1 by a p53-independent pathway. *Cancer Research*, 54:3391–3395.
- Mitchell, D. and Hartman, P. 1990. The regulation of DNA repair during development. *BioEssays*, 12:74.
- Miura, M., Nakamura, S., Sasaki, T., Takasaki, Y., Shiomi, T., and Yamaizumi, M. 1996. Roles of XPG and XPF/ERCC1 endonucleases in UV-induced immunostaining of PCNA in fibroblasts. *Experimental Cell Research*, 226(1):126–132.
- Moolenaar, G. F., Uiterkamp, R. S., Zwiijnenburg, D. A., and Goosen, N. 1998. The c-terminal region of the escherichia coli UvrC protein, which is homologous to the c-terminal region of the human ERCC1 protein, is involved in DNA binding and 5'-incision. *Nucleic Acids Research*, 26(2):462–468.
- Moore, P., Bose, K., Rabkin, S., and Strauss, B. 1981. Sites of termination of in vitro DNA synthesis in ultra-violet- and N-acetylaminofluorene-treated ϕ X174

- templates by prokaryotic and eukaryotic DNA polymerases. *Proceedings of the National Academy of Sciences of the United States of America*, 78:110–114.
- Moore, R., Redhead, N., Selfridge, J., Hope, J., Manson, J., and Melton, D. 1995. Double replacement gene targeting for the production of a series of mouse strains with different prion protein gene alterations. *Biotechnology*, 13:999–1004.
- Moriwaki, S.-I., Ray, S., Tarone, R., Kraemer, K., and Grossman, L. 1996. The effect of donor age on the processing of UV-damaged DNA by cultured human cells: Reduced DNA repair capacity and increased DNA mutability. *Mutation Research*, 364:117–123.
- Mu, D., Hsu, D. S., and Sancar, A. 1996. Reaction-mechanism of human DNA-repair excision nuclease. *Journal of Biological Chemistry*, 271(14):8285–8294.
- Murray, A. 1994. Cell cycle checkpoints. *Current Opinion in Cell Biology*, 6:872–876.
- Nagai, A., Saijo, M., Kuraoka, I., Matsuda, T., Kodo, N., Nakatsu, Y., Mimaki, T., Mino, M., Biggerstaff, M., Wood, R. D., Sijbers, A., Hoeijmakers, J. H. J., and Tanaka, K. 1995. Enhancement of damage-specific DNA-binding of XPA by interaction with the ERCC1 DNA-repair protein. *Biochemical and Biophysical Research Communications*, 211(3):960–966.
- Nakane, H., Takeuchi, S., Yuba, S., Saijo, M., Nakatsu, Y., Murai, H., Nakatsuru, Y., Ishikawa, T., Hirota, S., Kitamura, Y., Kato, Y., Tsunoda, Y., Miyauchi, H., Horio, T., Tokunaga, T., Matsunaga, T., Nikaido, O., Nishimune, Y., Okada, Y., and Tanaka, K. 1995. High-incidence of ultraviolet-b-induced or chemical-carcinogen-induced skin tumors in mice lacking the xeroderma-pigmentosum group-a gene. *Nature*, 377(6545):165–168.
- Nakanishi, M., Robetorye, R., Pereiras-Smith, O., and Smith, J. 1995. The C-terminal region of p21(Sdi1/Waf1/Cip1) is involved in proliferating cell nuclear antigen-binding but does not appear to be required for growth-inhibition. *Journal Of Biological Chemistry*, 270:17060–17063.
- Nance, M. and Berry, S. 1992. Cockayne syndrome: review of 140 cases. *The American Journal of Medical Genetics*, 42:68–84.
- Napolitano, R. L., Lambert, I. B., and Fuchs, R. P. P. 1997. Sos factors involved in translesion synthesis. *Proceedings of the National Academy of Sciences of the United States of America*, 94(11):5733–5738.
- Navas, T., Zhou, Z., and Elledge, S. 1995. DNA-polymerase-epsilon links the DNA-replication machinery to the s- phase checkpoint. *Cell*, 80:29–39.
- Nelson, J. R., Lawrence, C. W., and Hinkle, D. C. 1996. Thymine-thymine dimer bypass by yeast DNA polymerase ζ . *Science*, 272:1646–1649.
- Nelson, W. and Kastan, M. 1994. DNA strand breaks: the DNA template alterations that trigger p53-dependent DNA damage response pathways. *Molecular and Cellular Biology*, 14(3):1815–1823.
- Nichols, A. and Sancar, A. 1992. Purification of PCNA as a nucleotide excision repair protein. *Nucleic Acids Research*, 20:U3559–U3564.
- Nickoloff, J. and Hoekstra, M., editors 1998. *DNA damage and repair*. Humana Press Inc., Totowa, NJ.
- Niculescu III, A., Chen, X., Smeets, M., Hengst, L., Prives, C., and Reed, S. 1998. Effects of p21^{Cip1/Waf1} at both the G1/S and the G2/M cell cycle transitions: pRb is a critical determinant in blocking DNA replication and in preventing endoreplication. *Molecular and Cellular Biology*, 18:629–643.
- Nocentini, S., Coin, F., Saijo, M., Tanaka, K., and Egly, J. M. 1997. DNA damage

- recognition by XPA protein promotes efficient recruitment of transcription factor ii h. *Journal of Biological Chemistry*, 272(37):22991–22994.
- Noda, A., Ning, Y., Venable, S. F., Pereira-Smith, O. M., and Smith, J. R. 1994. Cloning of senescent cell-derived inhibitors of DNA-synthesis using an expression screen. *Experimental Cell Research*, 211(1):90–98.
- O'Connell, M. and Nurse, P. 1994. How cells know they are in G1 or G2. *Current Opinion in Cell Biology*, 6:867–871.
- O'Donovan, A., Davies, A. A., Moggs, J. G., West, S. C., and Wood, R. D. 1994. XPG endonuclease makes the 3' incision in human DNA nucleotide excision-repair. *Nature*, 371(6496):432–435.
- Orren, D., Petersen, L., and Bohr, V. 1995. A UV-responsive G2 checkpoint in rodent cells. *Molecular and Cellular Biology*, 15(7):3722–3730.
- Painter, R. 1985. Inhibition and recovery of DNA synthesis in human cells after exposure to ultraviolet light. *Mutation Research*, 145:63–69.
- Painter, R. 1986. Inhibition of mammalian cell DNA synthesis by ionizing radiation. *International Journal of Radiation Biology*, 49:771–781.
- Painter, R. and Young, B. 1976. Formation of nascent DNA during inhibition of replicon initiation in mammalian cells. *Biochimica Et Biophysica Acta*, 418:146–153.
- Park, C. H., Bessho, T., Matsunaga, T., and Sancar, A. 1995. Purification and characterization of the XPF-ERCC1 complex of human DNA-repair excision nuclease. *Journal of Biological Chemistry*, 270(39):22657–22660.
- Park, C. H. and Sancar, A. 1994. Formation of a ternary complex by human XPA, ERCC1, and ERCC4(XPF) excision-repair proteins. *Proceedings of the National Academy of Sciences of the United States of America*, 91(11):5017–5021.
- Park, S. and Cleaver, J. 1979. Postreplication repair: Question of its definition and possible alteration in XP cell strains. *Proceedings of the National Academy of Sciences of the United States of America*, 76:3927–3931.
- Parker, S., Eichele, G., Zhang, P., Rawls, A., Sands, A., Bradley, A., Olson, E., Harper, J., and Elledge, S. 1995. p53-independent expression of p21(CIP1) in muscle and other terminally differentiating cells. *Science*, 267:1024–1027.
- Pellegata, N. S., Antoniono, R. J., Redpath, J. L., and Stanbridge, E. J. 1996. DNA damage and p53-mediated cell cycle arrest: A reevaluation. *Proceedings of the National Academy of Sciences of the United States of America*, 93:15209–15214.
- Polyak, K., Hamilton, S. R., Vogelstein, B., and Kinzler, K. W. 1996. Early alteration of cell-cycle-regulated gene expression in colorectal neoplasia. *American Journal of Pathology*, 149(2):381–387.
- Poon, R. Y. C. and Hunter, T. 1998. Expression of a novel form of p21(Cip1/Waf1) in UV-irradiated and transformed cells. *Oncogene*, 16(10):1333–1343.
- Prakash, K., Wang, Z., and Friedberg, E. 1996. DNA repair and transcription. *Current Opinion in Genetics & Development*, 6:146–150.
- Rathmell, W. K., Kaufmann, W. K., Hurt, J. C., Byrd, L. L., and Chu, G. 1997. DNA-dependent protein kinase is not required for accumulation of p53 or cell cycle arrest after DNA damage. *Cancer Research*, 57(1):68–74.
- Reardon, J., Thompson, L., and Sancar, A. 1993. Excision repair in man and the molecular basis of xeroderma pigmentosum syndrome. *Cold Spring Harbor Symposium in Quantitative Biology*, 58:605–617.
- Reed, K. C. and Mann, D. A. 1985. Rapid transfer of DNA from agarose gels to nylon

- membranes. *Nucleic Acids Research*, 13(20):7207-7221.
- Reinke, V. and Lozano, G. 1997. Differential activation of p53 targets in cells treated with ultraviolet radiation that undergo both apoptosis and growth arrest. *Radiation Research*, 148(2):115-122.
- Roberts, J., Koff, A., Polyak, K., Firpo, E., Collins, S., Ohtsubo, M., and Massagué, J. 1994. Cyclins, cdks, and cyclin kinase inhibitors. *Cold Spring Harbor Symposia On Quantitative Biology*, 59:31-38.
- Robins, P., Jones, C., Biggerstaff, M., Lindahl, T., and Wodd, R. 1991. Complementation of DNA repair in xeroderma pigmentosum group A cell extracts by a protein with affinity for damaged DNA. *EMBO Journal*, 10:3913-3921.
- Rodel, C., Jupitz, T., and Schmidt, H. 1997. Complementation of the DNA repair-deficient swi10 mutant of fission yeast by the human ERCC1 gene. *Nucleic Acids Research*, 25(14):2823-2827.
- Rodel, C., Kirchhoff, S., and Schmidt, H. 1992. The protein-sequence and some intron positions are conserved between the switching gene swi10 of *Schizosaccharomyces pombe* and the human excision repair gene ERCC1. *Nucleic Acids Research*, 20(23):6347-6353.
- Rolig, R. L., Adair, G. M., and Nairn, R. S. 1995. Functional-characterization of ERCC1 in recombination and repair. *Journal of Cellular Biochemistry*, S21A SIA:284-284.
- Rosenbloom, A. L., Kappy, M. S., Debusk, F. L., Francis, G. L., Philpot, T. J., and Maclaren, N. K. 1983. Progeria - insulin resistance and hyperglycemia. *Journal of Pediatrics*, 102(3):400-402.
- Rowley, R. 1998. Mammalian cell-cycle responses to dna-damaging agents. In (Nickoloff and Hoekstra, 1998), pages 465-486.
- Rudé, J. and Friedberg, E. 1977. Semi-conservative deoxyribonucleic acid synthesis in unirradiated and ultraviolet-irradiated xeroderma pigmentosum and normal human skin fibroblasts. *Mutation Research*, 42:433-442.
- Rupp, W. and Howard-Flanders, P. 1968. Discontinuities in the DNA synthesised in an excision-defective strain of *E. coli* following UV irradiation. *Journal of Molecular Biology*, 31:291-304.
- Ryan, A. J., Squires, S., Strutt, H. L., Evans, A., and Johnson, R. T. 1994. Different fates of camptothecin induced replication fork associated double strand breaks in mammalian cells. *Carcinogenesis*, 15(5):823-828.
- Ryan, A. J., Squires, S., Strutt, H. L., and Johnson, R. T. 1991. Camptothecin cytotoxicity in mammalian-cells is associated with the induction of persistent double strand breaks in replicating DNA. *Nucleic Acids Research*, 19(12):3295-3300.
- Saijo, M., Kuraoka, I., Masutani, C., Hanaoka, F., and Tanaka, K. 1996. Sequential binding of DNA repair proteins RPA and ERCC1 to XPA in vitro. *Nucleic Acids Research*, 24(23):4719-4724.
- Sakata, H., Takayama, H., Sharp, R., Rubin, J., Merlino, G., and LaRochelle, W. 1996. Hepatocyte growth factor/scatter factor overexpression induces growth, abnormal development, and tumor formation in transgenic mouse livers. *Cell Growth And Differentiation*, 7:1513-1523.
- Sancar, A. 1994. Mechanisms of DNA excision-repair. *Science*, 266:1954-1956.
- Sancar, A. and Tang, M. 1993. Nucleotide excision repair. *Photochemistry & Photobiology*, 57:905-921.
- Sanchez, Y. and Elledge, S. 1995. Stopped for repairs. *Bioessays*, 17:545-548.

- Sands, A. T., Abuin, A., Sanchez, A., Conti, C. J., and Bradley, A. 1995. High susceptibility to ultraviolet-induced carcinogenesis in mice lacking XPC. *Nature*, 377(6545):162-165.
- Sargent, R. G., Rolig, R. L., Kilburn, A. E., Adair, G. M., Wilson, J. H., and Nairn, R. S. 1997. Recombination-dependent deletion formation in mammalian cells deficient in the nucleotide excision repair gene ERCC1. *Proceedings of the National Academy of Sciences of the United States of America*, 94(24):13122-13127.
- Savio, M., Stivala, L., Scovassi, A., Bianchi, L., and Prosperi, E. 1996. P21(Waf1/Cip1) protein associates with the detergent-insoluble form of PCNA concomitantly with disassembly of PCNA at nucleotide excision-repair sites. *Oncogene*, 13:1591-1598.
- Schaeffer, L., Roy, R., Humbert, S., Moncollin, V., Vermeulen, W., Hoeijmakers, J., Chambon, P., and Egly, J.-M. 1993. DNA repair helicase: a component of BTF2(TFIIH) basic transcription factor. *Science*, 260:58-63.
- Schauber, C., Chen, L., Tongaonkar, P., Vega, I., Lambertson, D., Potts, W., and Madura, K. 1998. Rad23 links DNA repair to the ubiquitin/proteasome pathway. *Nature*, 391(6668):715-718.
- Sekelski, J., McKim, K., Chin, G., and Hawley, R. 1995. The drosophila meiotic recombination gene mei-9 encodes a homologue of the yeast excision repair protein rad1. *Genetics*, 141:619-6276.
- Selter, H. and Montenarh, M. 1994. The emerging picture of p53. *International Journal Of Biochemistry*, 26:145-154.
- Sheikh, M. S., Chen, Y. Q., Smith, M. L., and Fornace, A. J. 1997. Role of p21(Waf1/Cip1/sd1) in cell death and DNA repair as studied using a tetracycline-inducible system in p53-deficient cells. *Oncogene*, 14(15):1875-1882.
- Shigemasa, K., Hu, C., West, C. M., Moon, S. H., Parham, G. P., Parmley, T. H., Korourian, S., Baker, V. V., and O'Brien, T. J. 1997. p21: a monitor of p53 dysfunction in ovarian neoplasia. *International Journal of Gynecological Cancer*, 7(4):296-303.
- Shiloh, Y., Tabor, E., and Becker, Y. 1985. *In vitro* phenotype of ataxia telangiectasia (AT) fibroblast strains: Clues to the nature of the 'AT DNA lesion' and the nuclear defect in AT. In (Gatti and Swift, 1985), pages 111-121.
- Shimizu, A., Nishida, J., Ueoka, Y., Kato, K., Hachiya, T., Kuriaki, Y., and Wake, N. 1998. Cyclin G contributes to G2/M arrest of cells in response to DNA damage. *Biochemical and Biophysical Research Communications*, 242(3):529-533.
- Sijbers, A., Vanderspek, P. J., Odijk, H., Vandenberg, J., van Duin, M., Westerveld, A., Jaspers, N. G. J., Bootsma, D., and Hoeijmakers, J. H. J. 1996a. Mutational analysis of the human nucleotide excision-repair gene ERCC1. *Nucleic Acids Research*, 24(17):3370-3380.
- Sijbers, A. M., Delaat, W. L., Ariza, R. R., Biggerstaff, M., Wei, Y. F., Moggs, J. G., Carter, K. C., Shell, B. K., Evans, E., Dejong, M. C., Rademakers, S., Derooij, J., Jaspers, N. G. J., Hoeijmakers, J. H. J., and Wood, R. D. 1996b. Xeroderma-pigmentosum group-f caused by a defect in a structure-specific DNA-repair endonuclease. *Cell*, 86(5):811-822.
- Smith, M., Chen, I., Zhan, Q., Bae, I., Chen, C., Gilmer, T., Kastan, M., O'Connor, P., and Fornace, A. 1994. Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. *Science*, 266:1376-1380.
- Smith, M., Chen, I., Zhan, Q., O'Connor, P., and Fornace, A. 1995. Involvement of the p53 tumor-suppressor in repair of UV-type DNA- damage. *Oncogene*, 10:1053-1059.

- Smith, M. and Fornace, A. 1996. Mammalian DNA damage-inducible genes associated with growth arrest and apoptosis. *Mutation Research-Reviews In Genetic Toxicology*, 340:109-124.
- Soames, A., Lavender, D., Foster, J., Williams, S., Wheeldon, E., and Fornace, A. 1994. Image analysis of bromodeoxyuridine (BrdU) staining for measurement of S-phase in rat and mouse liver. *The Journal of Histochemistry and Cytochemistry*, 42(7):939-944.
- Spivak, G. and Hanawalt, P. 1992. Translesion DNA-synthesis in the dihydrofolate-reductase domain of UV-irradiated CHO cells. *Biochemistry*, 31:6794-6800.
- Stamato, T. D. and Denko, N. 1990. Asymmetric field inversion gel-electrophoresis - a new method for detecting DNA double-strand breaks in mammalian-cells. *Radiation Research*, 121(2):196-205.
- Sugasawa, K., Ng, J. M. Y., Masutani, C., Iwai, S., vanderSpek, P. J., Eker, A. P. M., Hanaoka, F., Bootsma, D., and Hoeijmakers, J. H. J. 1998. Xeroderma pigmentosum group C protein complex is the initiator of global genome nucleotide excision repair. *Molecular Cell*, 2(2):223-232.
- Sugasawa, K., Ng, J. M. Y., Masutani, C., Maekawa, T., Uchida, A., vanderSpek, P. J., Eker, A. P. M., Rademakers, S., Visser, C., Aboussekhra, A., Wood, R. D., Hanaoka, F., Bootsma, D., and Hoeijmakers, J. H. J. 1997. Two human homologs of rad23 are functionally interchangeable in complex formation and stimulation of xpc repair activity. *Molecular and Cellular Biology*, 17(12):6924-6931.
- Sugita, K., Suzuki, N., Fujii, K., and Niimi, H. 1995. Reduction of unscheduled DNA-synthesis and plasminogen-activator activity in Hutchinson-Gilford fibroblasts during passaging in-vitro - partial correction by interferon-beta. *Mutation Research-DNA Genetic Instability and Aging*, 316(3):133-138.
- Sun, X., Shimizu, H., and Yamamoto, K. 1995. Identification of a novel p53 promoter element involved in genotoxic stress-inducible p53 gene-expression. *Molecular And Cellular Biology*, 15:4489-4496.
- Takahashi, K. and Suzuki, K. 1994. Nuclear exclusion of p53 controls DNA-synthesis through reduction in the expression of p21 inhibitor of cyclin-dependent kinase-2. *International Journal Of Oncology*, 5:1059-1064.
- Takayama, K., Salazar, E. P., Broughton, B. C., Lehmann, A. R., Sarasin, A., Thompson, L. H., and Weber, C. A. 1996. Defects in the DNA-repair and transcription gene ERCC2(XPD) in trichothiodystrophy. *American Journal of Human Genetics*, 58(2):263-270.
- Thompson, L. 1998. Nucleotide excision repair - its relation to human disease. In (Nickoloff and Hoekstra, 1998), pages 335-393.
- Thompson, L., Brookman, K., Weber, C., Salazar, E., Reardon, J., Sancar, A., Deng, Z., and Siciliano, M. 1994. Molecular-cloning of the human nucleotide-excision-repair gene ERCC4. *Proceedings Of The National Academy Of Sciences Of The United States Of America*, 91:6855-6859.
- Thompson, L., Busch, D., Brookman, K., Mooney, C., and Glaser, P. 1981. Genetic diversity of UV-sensitive DNA-repair mutants of chinese hamster ovary cells. *Proceedings Of The National Academy Of Sciences Of The United States Of America*, 78:3734-3737.
- Thompson, L., Fong, S., and Brookman, K. 1980. Validation of conditions for efficient detection of HPRT and APRT mutations in suspension-cultured chinese hamster ovary cells. *Mutation Research*, 74:21-36.

- Tomkinson, A. E., Bardwell, A. J., Bardwell, L., Tappe, N. J., and Friedberg, E. C. 1993. Yeast DNA-repair and recombination proteins rad1 and rad10 constitute a single-stranded-DNA endonuclease. *Nature*, 362(6423):860-862.
- Tomkinson, A. E., Lasko, D. D., Daly, G., and Lindahl, T. 1990. Mammalian DNA ligases - catalytic domain and size of DNA Ligase-I. *Journal of Biological Chemistry*, 265(21):12611-12617.
- Tomkinson, A. E. and Levin, D. 1997. Mammalian DNA ligases. *BioEssays*, 19(10):893-901.
- Tornaletti, S. and Hanawalt, P. C. 1999. Effect of DNA lesions on transcription elongation. *Biochimie*, 81(1-2):139-146.
- Usui, T., Yoshida, M., Abe, K., Osada, H., Isono, K., and Beppu, T. 1991. Uncoupled cell cycle without mitosis induced by a protein kinase inhibitor, k-252a. *The Journal of Cell Biology*, 115(5):1275-1282.
- van Duin, M., Dewit, J., Odijk, H., Westerveld, A., Yasui, A., Koken, M., Hoeijmakers, J., and Bootsma, D. 1986. Molecular characterization of the human excision repair gene ERCC-1 - cDNA cloning and amino-acid homology with the yeast DNA- repair gene rad10. *Cell*, 44:913-923.
- van Duin, M., Janssen, J., Dewit, J., Hoeijmakers, J., Thompson, L., Bootsma, D., and Westerveld, A. 1988. Transfection of the cloned human excision repair gene ERCC-1 to UV- sensitive CHO mutants only corrects the repair defect in complementation group-2 mutants. *Mutation Research*, 193:123-130.
- van Duin, M., Koken, M., Varentol, J., Tendijke, P., Odijk, H., Westerveld, A., Bootsma, D., and Hoeijmakers, J. 1987. Genomic characterization of the human DNA excision repair gene ERCC-1. *Nucleic Acids Research*, 15:9195-9213.
- van Duin, M., Vredeveltd, G., Mayne, L., Odijk, H., Vermeulen, W., Klein, B., Weeda, G., Hoeijmakers, J., Bootsma, D., and Westerveld, A. 1989. The cloned human DNA excision repair gene ERCC-1 fails to correct xeroderma pigmentosum complementation group-A through group-I. *Mutation Research*, 217:83-92.
- van Sloun, P. P. H., Romeijn, R. J., and Eeken, J. C. J. 1999. Molecular cloning, expression and chromosomal localisation of the mouse rev3l gene, encoding the catalytic subunit of polymerase zeta. *Mutation Research-DNA Repair*, 433(2):109-116.
- van Vuuren, A. J., Appeldoorn, E., Odijk, H., Humbert, S., Moncollin, V., Eker, A. P. M., Jaspers, N. G. J., Egly, J. M., and Hoeijmakers, J. H. J. 1995. Partial characterization of the DNA-repair protein complex, containing the ERCC1, ERCC4, ERCC11 and XPF correcting activities. *Mutation Research-DNA Repair*, 337(1):25-39.
- van Vuuren, A. J., Appeldoorn, E., Odijk, H., Yasui, A., Jaspers, N. G. J., Bootsma, D., and Hoeijmakers, J. H. J. 1993. Evidence for a repair enzyme complex involving ERCC1 and complementing activities of ERCC4, ERCC11 and xeroderma-pigmentosum group-f. *EMBO Journal*, 12(9):3693-3701.
- van Vuuren, A. J., Vermeulen, W., Ma, L., Weeda, G., Appeldoorn, E., Jaspers, N., van der Eb, A., Bootsma, D. Hoeijmakers, J., Humbert, S., Schaeffer, L., and Egly, J. 1994. Correction of xeroderma pigmentosum repair defect by basal transcription factor BTF2(TFIIH). *EMBO Journal*, 13:1645-1653.
- van Zeeland, A. and Filon, A. R. 1982. Post-replication repair:elongation of daughter strand DNA in UV-irradiated mammalian cells in culture. *Progress in Mutation Research*, 4:375-384.

- vanOijen, M., Medema, R. H., Slootweg, P. J., and Rijksen, G. 1998. Positivity of the proliferation marker ki-67 in noncycling cells. *American Journal of Clinical Pathology*, 110(1):24-31.
- Venema, J., Mullenders, L., and Natarajan, A. 1990. The genetic defect in cockayne's syndrome is associated with a defect in repair of UV-induced DNA damage in transcriptionally active DNA. *Proceedings of the National Academy of Sciences of the United States of America*, 87:4704-4711.
- Ventura, A. and Meneghini, R. 1984. Inhibition and recovery of the rate of DNA synthesis in V79 chinese hamster cells following ultraviolet light irradiation. *Mutation Research*, 131:81-88.
- Waga, S., Hannon, G., Beach, D., and Stillman, B. 1994. The p21 inhibitor of cyclin-dependent kinases controls DNA- replication by interaction with PCNA. *Nature*, 369:574-578.
- Wakasugi, M. and Sancar, A. 1998. Assembly, subunit composition, and footprint of human DNA repair excision nuclease. *Proceedings of the National Academy of Sciences of the United States of America*, 95(12):6669-6674.
- Waldman, T., Kinzler, K. W., and Vogelstein, B. 1995. P21 is necessary for the p53-mediated g(1) arrest in human cancer- cells. *Cancer Research*, 55(22):5187-5190.
- Waldman, T., Lengauer, C., Kinzler, K. W., and Vogelstein, B. 1996. Uncoupling of s phase and mitosis induced by anticancer agents in cells lacking p21. *Nature*, 381(6584):713-716.
- Walker, I. G. 1981. Alkaline sucrose sedimentation analysis as an indicator of repair capability of xeroderma pigmentosum fibroblasts for 4-nitroquinoline- 1-oxide damage. *Carcinogenesis*, 2(8):691-695.
- Wang, S. M., Nishigori, C., Yagi, T., and Takebe, H. 1991. Reduced DNA-repair in progeria cells and effects of gamma-ray irradiation on UV-induced unscheduled DNA-synthesis in normal and progeria cells. *Mutation Research*, 256(1):59-66.
- Wang, S. M., Nishigori, C., Zhang, J. M., and Takebe, H. 1990. Reduced DNA-repair capacity in cells originating from a progeria patient. *Mutation Research*, 237(5-6):253-257.
- Wang, X., Vermeulen, W., Coursen, J., Gibson, M., Lupold, S., Forrester, K., Xu, G., Elmore, L., Yeh, H., Hoeijmakers, J., and Harris, C. 1996. The XPB and XPD DNA helicases are components of the p53-mediated apoptosis pathway. *Genes & Development*, 10:1219-1232.
- Wang, X.-Q. and Ellem, K. 1994. Heterogeneity in the hela cell cycle response to UVC analysed by the brdurd two-parameter method. *Experimental Cell Research*, 212:176-189.
- Waters, R. 1979. Repair of DNA in replicated and unreplicated portions of the human genome. *Journal Of Molecular Biology*, 127:117-127.
- Webster, A., Barns, D., Arlett, C., Lehmann, A., and Lindahl, T. 1992. Growth retardation and immunodeficiency in a patient with mutations in the DNA ligase I gene. *The Lancet*, 339:1058-1059.
- Weeda, G., Donker, I., deWit, J., Morreau, H., Janssens, R., Vissers, C. J., Nigg, A., vanSteeg, H., Bootsma, D., and Hoeijmakers, J. H. J. 1997. Disruption of mouse ERCC1 results in a novel repair syndrome with growth failure, nuclear abnormalities and senescence. *Current Biology*, 7(6):427-439.
- Westerveld, A., Hoeijmakers, J., van Duin, M., Dewit, J., Odijk, H., Pastink, A., Wood, R., and Bootsma, D. 1984. Molecular-cloning of a human DNA-repair gene.

- Nature*, 310:425–429.
- Wilson, T., Grawunder, U., and Lieber, M. 1997. Yeast DNA ligase IV mediates non-homologous DNA end joining. *Nature*, 388(31):495–498.
- Wim, J. M., Vuillaume, M., Chrzanowska, K., Smeets, D., Sperling, K., and Hall, J. 1997. Nijmegen breakage syndrome cells fail to induce the p53-mediated DNA damage response following exposure to ionizing radiation. *Molecular and Cellular Biology*, 17(9):5016–5022.
- Wood, R. 1997a. DNA repair in eukaryotes. *Annual Review of Biochemistry*, 65:135–167.
- Wood, R. 1997b. Nucleotide excision repair in mammalian cells. *The Journal of Biological Chemistry*, 272(38):23465–23468.
- Wood, R. D. 1999. DNA damage recognition during nucleotide excision repair in mammalian cells. *Biochimie*, 81(1-2):39–44.
- Wood, R. D., Deviciana, M., and Pressontincknell, B. 1982. Post-irradiation properties of a UV-sensitive variant of CHO. *Photochemistry and Photobiology*, 36(2):169–174.
- Woodgate, R. and Levine, A. 1996. Damage-inducible mutagenesis: recent insights into the activities of the umu family of mutagenesis proteins. *Cancer Surv.*, 28:117–140.
- Wu, H., Wade, M., Krall, L., Grisham, J., Xiong, Y., and Vandyke, T. 1996. Targeted in-vivo expression of the cyclin-dependent kinase inhibitor p21 halts hepatocyte cell-cycle progression, postnatal liver development, and regeneration. *Genes & Development*, 10(3):245–260.
- Wu, L. and Levine, A. J. 1997. Differential regulation of the p21/waf-1 and mdm2 genes after high-dose UV irradiation: p53-dependent and p53-independent regulation of the mdm2 gene. *Molecular Medicine*, 3(7):441–451.
- Wu, Y., Huang, H., Miner, Z., and KuleszMartin, M. 1997. Activities and response to DNA damage of latent and active sequence-specific DNA binding forms of mouse p53. *Proceedings of the National Academy of Sciences of the United States of America*, 94(17):8982–8987.
- Wyllie, F., Haughton, M., Bond, J., Rowson, J., Jones, C., and Wynfordthomas, D. 1996. S-phase cell-cycle arrest following DNA-damage is independent of the p53/p21(Waf1) signaling pathway. *Oncogene*, 12:1077–1082.
- Xiao, W., Lechler, T., Chow, B., Fontanie, T., Agustus, M., Carter, K., and Wei, Y.-F. 1998. Identification, chromosomal mapping and tissue-specific expression of hrev3 encoding a putative human DNA polymerase ζ . *Carcinogenesis*, 19(5):945–949.
- Yagi, T., Wood, R. D., and Takebe, H. 1997. A low content of ERCC1 and a 120 kDa protein is a frequent feature of group F xeroderma pigmentosum fibroblast cells. *Mutagenesis*, 12(1):41–44.
- Yu, C. E., Oshima, J., and Fu, Y. H. 1996. Positional cloning of the werner's syndrome gene. *Science*, 272:258–262.
- Zdzienicka, M., Westerveld, A., Simons, J., and Bootsma, D. 1987. Biological and biochemical consequences of the human ERCC1 repair gene in a repair-deficient chinese-hamster ovary cell-line. *Mutation Research*, 181:353.
- Zebrower, M., Kieras, F. J., and Brown, W. T. 1986. Urinary hyaluronic-acid elevation in Hutchinson-Gilford progeria syndrome. *Mechanisms of Ageing and Development*, 35(1):39–46.
- Zhang, H., Hannon, G. J., Casso, D., and Beach, D. 1994. p21 is a component of active cell cycle kinases. *Cold Spring Harbor Symposia on Quantitative Biology*, 59:21–29.
- Zhang, W., Grasso, L., McClain, C., Gambel, A., Cha, Y., Travalii, S., Deis-

- seroth, A., and Mercer, W. 1995. p53-independent induction of WAF1/CIP1 in human leukemia-cells is correlated with growth arrest accompanying monocyte/macrophage differentiation. *Cancer Research*, 55(3):668-674.
- Ziegler, A., Jonason, A., Leffell, D., Simon, J., Sharma, H., Kimmelman, J., Remington, L., Jacks, T., and Brash, D. 1994. Sunburn and p53 in the onset of skin-cancer. *Nature*, 372:773-776.